University of Alberta

Relationship between Dietary Fat, Membrane Fatty Acid Composition and Function of the Photoreceptor Cells: Role of Very Long Chain (C24-C36) Polyenoic Fatty Acids

by

Miyoung Suh ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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Department of Agricultural, Food and Nutritional Science

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Relationship between Dietary Fat, Membrane Fatty Acid Composition and Function of the Photoreceptor Cells: Role of Very Long Chain (C24-C36) Polyenoic Fatty Acids** submitted by **Miyoung Suh** in partial fulfillment of the requirement for the degree of **Doctor of Philosophy** in **Nutrition and Metabolism.**

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ABSTRACT

The purpose of this study was to examine the relationship between dietary fat, membrane fatty acid composition and rhodopsin function. The effect of dietary fat on: 1) long and very long chain fatty acid (LCFA, VLCFA) composition in rod outer segments (ROS); 2) light exposed membrane fatty acid composition; 3) rhodopsin content, rhodopsin photolyzing kinetics, rhodopsin phosphorylation and rhodopsin regeneration; 4) fatty acid composition and rhodopsin function in the growing rat; 5) synthesis of LCFA and VLCFA using radiolabeled C20:5n-3 and C22:6n-3; was assessed.

ROS was isolated to measure fatty acid composition of phospholipids in animals fed diets containing a high n-3 or low n-3 fatty acid content. Diets high in n-3 fatty acids increased the levels of C22:5n-3 and C22:6n-3, while decreasing the C22:4n-6 and C22:5n-6 level in all phospholipid. N-6 and n-3 VLCFA of C24 to C34 with 4, 5 and 6 double bonds were found only in phosphatidylcholine (PC). Feeding a high n-3 fatty acid diet significantly reduced n-6 tetraenoic VLCFA. After 48 hrs of light exposure, animals fed a high n-3 fatty acid showed reduction in C22:6n-3 in PC and phosphatidylserine. Light treatment also reduced n-6 and n-3 VLCFA compared to the dark adapted group. Diet low in n-3 fatty acid increased rhodopsin content and rhodopsin phosphorylation compared to high n-3 diet in retina and ROS of dark adapted animals. However, low n-3 fatty acid diet causes greater rhodopsin loss after light exposure, resulting in less phosphorylation. Rhodopsin in animals fed a high n-3 fatty acid diet disappeared in a relatively short time after the visual cells were exposed to light. Rhodopsin regeneration measured *in vitro* was increased by feeding a high n-3 fatty acid diet. Developmental changes were characterized by decrease in C20:4n-6 and n-6 tetraenoic VLCFA in PC whereas C22:6n-3 and n-3 pentaenoic and hexaenoic VLCFA increased with age. Inclusion of small amount of C20:4n-6 and C22:6n-3 in diet increased both C20:4n-6 and C22:6n-3 levels in PC. Feeding C20:4n-6 or C22:6n-3 further increased the level of n-6 VLCFA and n-3 VLCFA respectively. The highest rhodopsin content occurred in the retina of animals fed diets containing C20:4n-6 and/or C22:6n-3. Rhodopsin in animals fed a C22:6n-3 diet photolyzed in a short time after light exposure to retina. After intravitreal injection of ^{3}H-20:5n-3 or ^{3}H-22:6n-3 into each eye, ^{3}H-C22:6n-3 remained in C22:6n-3, whereas ^{3}H-20:5n-3 was actively incorporated into pentaenoic and hexaenoic VLCFA.

It is concluded that LCFA, VLCFA and rhodopsin function of visual cells are influenced by both dietary fat fed and exposure to light. Feeding a high n-3 fatty acid diet may play a role in conserving rhodopsin during or after light exposure. The membrane fatty acid composition and rhodopsin content of developing photoreceptor cells are sensitive to dietary supply of C20:4n-6 and C22:6n-3. C20:5n-3 and C22:5n-3 are metabolically active precursors for synthesis of VLCFA but C22:6n-3 is incorporated directly into photoreceptors without further metabolism.

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Abbreviations

AA = arachidonic acid

ALD = adrenoleukodystrophy

ALDP = adrenoleukodystrophy protein

ATP = adenosine triphosphate

CDP = cytidine diphosphate

CoA = coenzyme A

CTP = cytidine triphosphate

DHA = docosahexaenoic acid

ERG = electroretinogram

GC-MS = gas liquid chromatography-mass spectrometry

GLC = gas liquid chromatography

LA = linoleic acid

LCFA = long chain fatty acid

n- = denotes the position of a double bond in a fatty acid from the methyl end

LNA = α-linolenic acid

NAD^+ = nicotineamide adenine dinucleotide

PL = phospholipid

PRCD = progressive rod-cone degeneration

P/S = the ratio of polyunsaturated to saturated fatty acids

RCS = royal college of surgeons

ROS = rod outer segment

SAS = statistical analysis system

SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM = standard error of the mean

TLC = thin layer chromatography

UV = ultra violet

VLCFA = very long chain fatty acid; defined as 24 carbon chain length and longer

Chapter I. INTRODUCTION

The characteristic fatty acid composition of phospholipids regulates in part the physiological properties of biomembranes. Membrane fatty acids vary with cell type, organ, stage of development and physiological state of the animal. Furthermore, the composition of phospholipids is affected by dietary fatty acid source which is directly related to the maintenance of membrane for its optimal function. The retina is an interesting membrane for study of lipid metabolism because of its unique lipid components and its active lipid metabolism. Recent understanding of the membrane lipid composition has been extended with the discovery of very long chain fatty acids with chain length over 22 carbons in retinal membranes. Lack of adequate information about these fatty acids including their metabolism and function leaves much to be elucidated.

The unique constituents of the retina are its highly enriched levels of docosahexaenoic acid in individual phospholipids, dipolyunsaturated phospholipid species and the presence of polyenoic very long chain fatty acids. Therefore, this chapter will focus on the lipid composition and metabolism of retina and factors related to its membrane changes including developmental variations, diet, light and disease.

A. VISION CELLS

Structure

Retina

The retina is the most critical structure in the visual sensory organ. It is a thin layer of neural tissue of highly complex structure which lines two-thirds of the posterior of the eye cup. The retina develops embryologically from invagination of the optic vesicle which is an extension of the primitive forebrain (neural cells) and its structure is that of a layer of nerve cells interconnected through synapses (Lettivin et al., 1969). The retina consists of pigment epithelium, photoreceptors (rod & cone cells), four types of interneuron networks (bipolar, ganglion, horizontal & amacrine cells), and their fibers. In visual processing

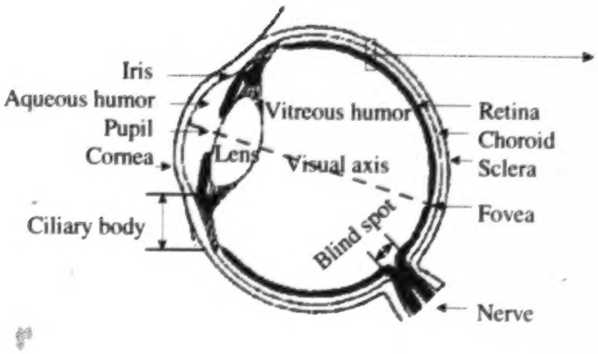
Iris
Aqueous humor
Pupil
Cornea
Ciliary body
Vitreous humor
Lens
Visual axis
Blind spot
Retina
Choroid
Sclera
Fovea
Nerve

Figure I-1. Major structure of eye. Hatched line indicates direction of light entering into eye.

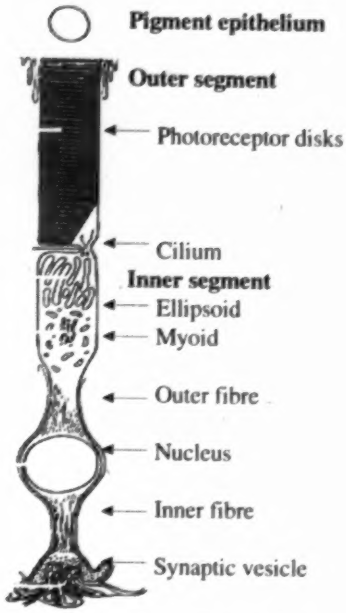
Pigment epithelium
Outer segment
Photoreceptor disks
Cilium
Inner segment
Ellipsoid
Myoid
Outer fibre
Nucleus
Inner fibre
Synaptic vesicle

Figure I-2. Organization of rod cell in the vertebrate retina. Rod outer segment was filled with a stack of disc-shaped double membrane lipid bilayer.

the retina plays the key role of light conversion into nervous excitation. When light enters the eyeball, it passes through the cornea, aqueous humor, lens, vitreous humor and various retinal layers before striking the photoreceptors (Figure I-1). After absorbing the light photon and generating electrical signals, the receptors send these signals encoded to bipolar cells and hence to ganglion cells, which in turn send visual information along the optic nerve to the visual cortex of the brain. Horizontal and amacrine cells make lateral connections at a level near the receptors and the ganglion cells respectively. All of these cells are held together by neuroglia cells, the Muller cells, which span the full thickness of the retina.

The pigment epithelium is a melanin-rich monolayer of cells located between photoreceptors and the choroidal blood supply that controls the flow of nutrients and metabolites into and out of the neurosensory retina (Zauberman, 1979; Cohen, 1965). The pigment epithelium also plays a macrophage like role. It phagocytes and degrades rod and cone outer segment discs which are continually being shed from the outer tips of the photoreceptor cells, implying that it participates in photoreceptor renewal (Ishikawa & Yamada, 1970; Young, 1976; Gordon & Bazan, 1993) (The details are described in the section C of this chapter). The blood supply of the retina for exchange of nutrients is derived from two independent sources. One is the choroidal system for serving the outer retinal layer and is located between the sclera and retinal pigment epithelium (RPE) without penetrating it into the retina. A second blood supply is the retinal arterial system which serves as a source of nutrients for the inner retinal layer that penetrates the photoreceptor cell layer at the blind spot.

Photoreceptors

The photoreceptors are the actual site of light photon absorption. Photoreceptors are highly polarized cells with pigment molecules. There are two physiologically distinct visual cell types, the rods and cones. Functionally, rods are responsible for dim light and peripheral vision while cones are specialized for bright light and colour vision. Both cell types have synaptic endings, nuclei, a metabolically active inner segment and a light sensitive outer segment. The inner segment is divided into two regions: one is the ellipsoid region which is a dense aggregation of mitochondria and the other region is a myoid which contains ribosomes, rough endoplasmic reticulum and golgi apparatus, participating in the synthesis of protein, lipid and other molecules. The outer segment consists of a dense system of parallel membranes in which photosensitive pigments are concentrated. Since many studies have been done on rod cells for understanding photoreceptor cell metabolism, the following discussion will emphasize the rod cell.

Rod Outer Segments (ROS)

The typical organization of rod cells is depicted in Figure I-2. The ROS is the major photon capturing device which consists of a stack of disc-shaped double membrane lipid bilayers derived by the evagination of the outer segment plasma membrane (Steinberg, 1980). Electron microscopic studies indicate that the disc membrane is symmetrical in cross section with the hydrocarbon region of the lipid in the center of the membrane. The visual pigments are densely occupied as intrinsic, transmembrane proteins within the disc membrane (Korenbrot, 1985). The opsin, particularly rhodopsin, is the predominant membrane protein and has carbohydrate side chains. In bovine ROS, 85% of the protein is rhodopsin (Daeman, 1973) with a concentration as high as 3.5 mM (Hargrave & McDowell, 1992). These membrane contents allow the cell to induce proper orientation of the protein and flexing and twisting. ROS is connected to the inner segment by a hollow cilium which serves as a supply channel between two parts of the cell (Sjostrand, 1953).

Rhodopsin and Visual Cycle

The adaptation of animals to light exposure depends on the balance of bleaching and regeneration of rhodopsin. Rhodopsin is firmly embedded in the hydrophobic lipid bilayer of the disk and contains the light sensitive chromophore 11-*cis* retinaldehyde. The N-terminus of the protein contains oligosaccharide moieties and is exposed to the intradiscal space. The C-terminus facing to cytoplasmic spaces contains the sites for phosphorylation and binding and activating the G-protein.

Illumination of rhodopsin initiates the photoisomerization of 11-*cis* retinal to all-trans configuration, resulting in rhodopsin undergoing a series of changes such as bathorhodopsin, lumirhodopsin, metarhodopsin I and metarhodopsin II. From the transformation of metarhodopsin I to metarhodopsin II (R*), its deprotonated Schiff base is hydrolyzed to release all-*trans* retinal and to reduce to all-*trans* retinol (Figure I-3). The all-*trans* alcohol form is then transported to the retinal pigment epithelium, where it is esterified to fatty acid, and isomerized back to the 11-*cis* configuration. After isomerization, the 11-*cis* retinol is oxidized to 11-*cis* retinaldehyde, which is then transported back to the photoreceptor cells. Then it can combine with opsin to regenerate

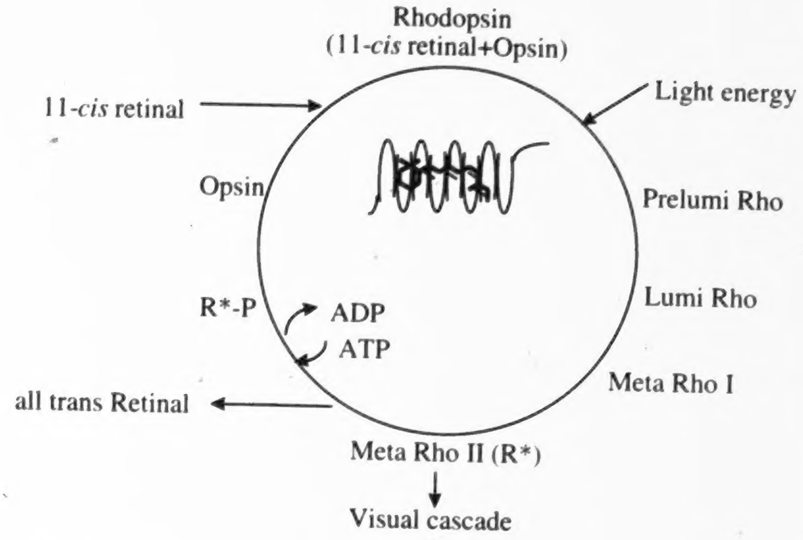
Rhodopsin
(11-*cis* retinal+Opsin)
11-*cis* retinal
Light energy
Opsin
Prelumi Rho
R*-P
ADP
ATP
Lumi Rho
Meta Rho I
all trans Retinal
Meta Rho II (R*)
Visual cascade

Figure I-3. Rhodopsin photochemistry

a functional visual pigment molecule. This cycle is the major contributor of rhodopsin regeneration during dark adaptation (Jones et al., 1989). It can be concluded that the regeneration depends on the availability of 11-*cis* retinal and the ability of the opsin to condense with the chromophore (Zorn & Futterman, 1971; Cocozza & Ostroy, 1987). Externally applied 11-*cis* retinal to the light adapted retinas also increases rhodopsin regeneration (Pepperberg et al., 1976; Jones et al., 1989; Huang et al., 1990). Delipidated rhodopsin is not able to regenerate rhodopsin, but the regeneration is restored by addition of phospholipids (Shichi, 1971) and especially phosphatidylethanolamine (Zorn & Futterman, 1971). Rod outer segment treated with NH_4OH to remove C16:0 results in reduced pigment regeneration (Morrison et al., 1991). Bush et al. (1994) reported rats fed a n-3 fatty acid diet exhibit slower rhodopsin regeneration than rats fed a soybean oil. This suggests that rhodopsin regeneration requires specific components and lipid may be one of them. Rats deficient in vitamin A exhibit both a decreased level of rhodopsin and substantially decreased visual sensitivity (Noell et al., 1971).

Photoactivated rhodopsin is inactivated by phosphorylation in the C-terminal of rhodopsin (Figure I-3). This reaction is catalayzed by rhodopsin kinase (molecular weight, 68,000). The target of rhodopsin kinase is bleached rhodopsin (Kuhn et al., 1978; Frank & Buzney, 1975). However, it can also phosphorylate nonbleached rhodopsin (Bownds et al., 1974; Chen et al., 1995) but this phosphorylation does not exceed 2% in frog retina (Binder et al., 1996). Rhodopsin kinase is dissociated from the ROS disk membrane in the dark, and is soluble in the cytoplasmic fluid between the disks (Lolley, 1983). Rhodopsin kinase binds to the surface of the ROS disk by light exposure and phosphorylates one to five serine/threonine residues located at C-terminal of rhodopsin (Ohguro et al., 1995; Sale et al., 1978). Phosphorylation is thought to inactivate bleached rhodopsin (Liebman & Pugh, 1980). Incorporated phosphates are removed slowly from rhodopsin in the dark by another enzyme, phosphoprotein phosphatase (PPP). The combined reactions of rhodopsin phosphorylation and dephosphorylation involve the hydrolysis of ATP to ADP and Pi. A role for phosphorylated rhodopsin in ROS metabolism or function is still not clear, but it may modulate the efficiency with which rhodopsin activates the hydrolysis of cyclic GMP (Lolley, 1983).

Visual Cell Renewal

The balance between the processes of cell formation and degradation is critical in living cells to maintain their characteristic functions. A unique feature of rods and cones is the capability of spending their energy not only on the initiation of visual messages but also on the renewal of their own molecules (Young, 1976). Repeated assembly of new discs at the base of the ROS is balanced by incessant shedding of the outer segment apex by means of membrane replacement and/or molecular replacement. Membrane replacement means the addition of newly formed membrane to old existing membranes. Molecular replacement involves exchange of individual membrane components with those which already exist in the membrane. Using an autoradiographic technique, Young (1976) and Bibb & Young

(1974a & 1974b) revealed the renewal mechanism of membrane constituents such as proteins and lipids.

Protein

Autoradiography shows that proteins undergo continuous turnover (Young & Droz, 1968). Following the injection of ^{3}H-amino acids into frog retina, radioactivity was initially incorporated into protein synthesized in the rough endoplasmic reticulum of the myoid. The radiolabelled product is then transported to the Golgi apparatus and then to the base of the outer segment. With increasing time, the radiolabelled product migrates as a band to the apex of the outer segment and is shed and eventually phagocytized by the retinal pigment epithelium. Using immunocytochemical techniques, La Vail et al. (1976) found that disc shedding is influenced by light. Shedding is minimal in the dark whereas it occurs vigorously soon after the onset of light. Papermaster and colleagues (1979) found that rhodopsin in vesicles is inserted into the basal folding of the plasma membrane and that these proteins are always bound to a membrane. Once incorporated into the rod disc, the rhodopsin is not replaced throughout the disc's lifetime. Young (1976) interpreted this as the process of membrane replacement.

Lipid

After injection of ^{3}H-glycerol into frog retina *in vivo*, the labelling profile of glycerol has been observed to be the same as protein membrane renewal (Bibb & Young, 1974b). Labelled glycerol backbone phospholipids are synthesized on the endoplasmic reticulum of the inner segment, and are transported to the base of the outer segment where membrane assembly is made by fusion of the vesicles with the plasma membrane (Anderson et al., 1980c; Bibb & Young, 1974b). These new phospholipid molecules become integral parts of the membrane and rapidly diffuse throughout the outer segment. When radiolabeled palmitic and docosahexaenoic acid and choline are used as precursors of the lipid components, these immediately appear throughout the cell and ROS, instead of being concentrated in the myoid part (Gordon & Bazan, 1990). This observation results from the exchange of choline and fatty acids with already situated old components of membrane phospholipid (De Turco et al., 1990 & 1991; Gordon & Bazan, 1990; Bibb & Young, 1974a; Anderson et al., 1980c). The half life of phosphatidylethanolamine and phophatidylcholine in the ROS synthesized from labelled glycerol was eighteen to nineteen days (Anderson et al., 1980a & 1980c). In the rhesus monkey, each rod assembles 80-90 new discs daily and completely renews its membranes every 9 to 12 days (Young, 1971). It has been reported that feeding a fat-free diet to rats causes impaired disc membrane renewal (Anderson et al., 1974). Unlike protein, therefore, phospholipid in the ROS undergoes both membrane replacement and molecular replacement (Young, 1976).

Lipid Composition

Whole retina

The lipid composition of the retina has been rather extensively investigated in an attempt to understand how the molecular architecture of the membranes underlies retinal function. It is well known that the retina is the richest source of lipid, mostly phospholipid, among any ocular tissues. Among the lipid (20% by dry weight of the retina), phospholipid comprises about two-thirds of the total lipid in the retina (Fliesler &Anderson, 1983) while cholesterol represents approximately 9 % of the total lipid (Fliesler & Schroepfer, 1982). In all subcellular fractions analyzed from the retina, phosphatidylcholine is the most prominent phospholipid class (Careaga & Bazan, 1981). The average phospholipid composition from several vertebrates are summarized in Table I-1. Phosphatidylcholine (47%), phosphatidylethanolamine (33%) and phosphatidylserine (9%) are the most abundant and make up approximately 90% of the phospholipid in the retina. Phosphatidylinositol (5%) and sphingomyelin (3%) are also minor phospholipid components. Ten to forty percent of ethanolamine containing phospholipid is in the form of plasmalogen in bovine and rabbit retina, respectively (Anderson et al., 1970).

Table I-1. The overall phospholipid composition of vertebrate retina and rod outer segment.

Phospholipid (mol %)	Retina	Rod outer segment
Phosphatidylcholine	46.6 ± 0.1	38.0 ± 2.9
Phosphatidylethanolamine	32.5 ± 0.7	39.3 ± 2.8
Phosphatidylserine	9.0 ± 0.4	14.4 ± 0.5
Phosphatidylinositol	4.9 ± 0.3	1.9 ± 0.2
Sphingomyelin	3.4 ± 0.5	1.2 ± 0.2

Values represent means ± S.E.M. The values of retina composition are an average from dog, pig, human, sheep, bovine and rabbit samples (Anderson et al., 1970; Nielsen et al., 1986). The values of rod outer segment composition are an average of rat, dog, frog, bovine and rabbit samples (Anderson & Risk, 1974; Anderson et al., 1975; Wetzel & O'Brien, 1986; Fliesler & Anderson, 1983).

The overall concentration of phospholipid in the retina is about 15 mg/g of wet tissue (Broekhuyse & Daeman, 1977).

Several researchers (Anderson et al.,1970; Nielson et al., 1986) have also carried out detailed fatty acid analysis, indicating that different phospholipids have distinct fatty acid compositions. The major polyunsaturated fatty acid in all phospholipid fractions is docosahexaenoic acid followed by arachidonic acid. The major saturated fatty acid in phosphatidylcholine is palmitic acid, whereas stearic acid is the major saturated fatty acid in phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin. Phosphatidylethanolamine and phosphatidylserine contain a relatively higher content of polyunsaturated fatty acids (42% and 34% respectively) as compared to phosphatidylcholine (13%) (Table I-2). The polyunsaturated fatty acid in phosphatidylinositol is mainly arachidonic acid (32%) and sphingomyelin contains 13% palmitic acid and 40% stearic acid but lacks polyunsaturated fatty acids.

Table I-2. The overall phospholipid and fatty acid composition of vertebrate retina

Phospholipid	PC	PE	PS	PI	SM
mol %	46.6 ± 1.0	32.5 ± 0.7	9.0 ± 0.4	4.7 ± 0.3	3.4 ± 0.5
Fatty acid (mol %)					
C16:0	42.5 ± 1.6	9.6 ± 0.9	6.2 ± 1.0	12.8 ± 1.5	23.0 ± 2.4
C18:0	19.3 ± 1.1	29.3 ± 1.2	40.6 ± 1.7	39.6 ± 0.8	48.4 ± 7.5
C18:1	22.7 ± 1.5	10.5 ± 1.0	17.0 ± 1.5	9.0 ± 0.9	10.2 ± 2.5
C20:4n-6	3.5 ± 0.5	9.5 ± 0.8	3.9 ± 0.7	32.5 ± 3.4	-
C22:6n-3	8.8 ± 1.0	27.4 ± 1.7	22.9 ± 2.4	2.4 ± 0.1	-
Σ SAT	63.2 ± 1.9	38.0 ± 2.5	47.3 ± 1.8	53.0 ± 2.0	84.5 ± 4.9
Σ PUFA	12.7 ± 2.4	41.9 ± 2.0	34.2 ± 4.2	34.2 ± 4.1	-

The values of phospholipid and fatty acid composition are an average of values from dog, pig, human, sheep, bovine and rabbit (Anderson et al., 1970 and Nielsen et al., 1986). Values represent mean ± S.E.M. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; Σ SAT, sum of saturated fatty acids; Σ PUFA, sum of polyunsaturated fatty acids.

Rod Outer Segments

The rod outer segment (ROS) is the only subcellular fraction of the retina in which the phospholipid and fatty acid composition has been extensively studied. ROS membranes (>95% disc membrane & <5% plasma membrane) consist of 60% protein and 40% lipid (Hitoshi, 1982). The phospholipids comprise about 85-90% of the total lipid of the ROS (Anderson & Andrews, 1982). The major neutral lipid is cholesterol which accounts for only 5-7 mol % of the total ROS lipid (Fliesler & Schroepfer, 1982). 1,2-diacylglycerol, phosphatidic acid and free fatty acids are minor constituents (Giusto et al., 1983). Compared to the entire retina, ROS has a higher level of phosphatidylserine (14%) and phosphatidylethanolamine (39%) and a lower level of phosphatidylcholine (38%). Phosphatidylinositol (2%) and sphingomyelin (1%) are minor components. The overall phospholipid composition of mammalian ROS is similar and is summarized in Table I-1. Using a novel pair of permeable and impermeable covalent modification reagents, the transmembrane distribution of phospholipid is asymmetric between the inner and outer monolayers in the ROS disc membranes (Miljanich et al., 1981). The aminophospholipids, phosphatidylethanolamine (73-87% of disc PE) and phosphatidylserine (77-88% of disc PS) are located in the outer disc membrane monolayer and most of the phosphatidylcholine (65-100% of disc PC) is on the inner membrane surface (Miljanich, 1981; Sklar et al., 1979). These results provide insight into the possible role of phospholipids in membrane function.

The most striking feature of the fatty acids of the ROS membrane is their high content of polyunsaturated fatty acids which comprises about half of the fatty acids in the ROS membrane (Anderson et al., 1974) (Table I-3). Similarly, large amounts of polyunsaturated fatty acids have been found in synaptic plasma membranes among the brain subcellular organelles. The most abundant polyunsaturated fatty acid is docosahexaenoic acid. In frog and bovine ROS, approximately 60-70% of the total polyunsaturated fatty acid is accounted for by docosahexaenoic acid, the major fatty acid in phosphatidylethanolamine and phosphatidylserine (Anderson et al., 1974; Anderson &

TableI-3. The overall phospholipid and fatty acid composition of vertebrate rod outer segment.

Phospholipid	PC	PE	PS	PI	SM
mol %	38.0 ± 2.9	39.3 ± 2.8	14.4 ± 0.5	1.9 ± 0.2	1.2 ± 0.2
Fatty acid (mol %)					
C16:0	31.5 ± 2.8	8.7 ± 2.5	2.6 ± 0.8	16.4 ± 1.8	-
C18:0	22.4 ± 0.5	20.3 ± 4.9	22.3 ± 2.5	36.9 ± 4.8	-
C18:1	7.8 ± 1.4	7.1 ± 1.1	3.4 ± 1.4	9.8 ± 1.7	-
C20:4n-6	2.8 ± 0.4	4.9 ± 0.7	2.0 ± 0.6	17.7 ± 0.1	-
C22:6n-3	23.0 ± 3.8	40.0 ± 7.2	38.5 ± 8.2	9.8 ± 7.0	-
Σ SAT	55.1 ± 2.3	29.0 ± 7.0	26.0 ± 2.3	53.3 ± 2.9	-
Σ PUFA	32.8 ± 2.0	62.1 ± 6.1	61.0 ± 2.3	31.2 ± 4.8	-

The values of phospholipid composition are an average of values from rat, dog, frog, bovine and rabbit samples (Anderson & Risk, 1974; Anderson et al., 1975; Nielsen et al., 1986; Wetzel & O'Brien, 1986 and Fliesler & Anderson, 1983). The values of fatty acid composition of individual phospholipids are from frog, bovine and rabbit samples (Anderson & Risk, 1974; Anderson et al., 1975; Nielsen et al., 1986 and Wiegand & Anderson, 1983). Values represent mean ± S.E.M. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; Σ SAT, sum of saturated fatty acids; Σ PUFA, sum of polyunsaturated fatty acids.

Maude, 1970). The major saturated fatty acid in individual phospholipid in ROS is similar to fatty acid found in retina. These results combined with the phospholipid bilayer distribution indicate that the total inner membrane monolayer contains more saturated fatty acids than the outer monolayer (Miljanich et al., 1981).

The classical positional distribution of fatty acids of phospholipids has been determined; saturated fatty acids esterified primarily at the *sn*-1 position while polyunsaturated fatty acids are found primarily at the *sn*-2 position of the glycerol moiety (Anderson & Sperling, 1971). However, recent studies have described a different molecular species of phospholipids from bovine ROS and microsomes, indicating the presence of dipolyunsaturated fatty acid phospholipids (Aveldano, 1989; Miljanich et al., 1979; Aveldano & Bazan, 1983; Choe & Anderson, 1990). It is interesting that in these species long chain polyunsaturated fatty acids are esterified to both the *sn*-1 and *sn*-2 positions of the glycerol backbone. These species are shown to be prominent components of the major lipids from cattle and frog retinal ROS (Miljanich et al., 1979; Aveldano & Bazan, 1983; Wiegand & Anderson, 1983). As ascertained by argentation thin layer chromatography of acetyldiglyceride derivatives, a method that separates groups according to their total number of double bonds, bovine ROS contain one-third of the phospholipid as dipolyunsaturated phospholipids or supraenoic species (Aveldano & Bazan, 1983; Aveldano, 1989). These phospholipids represent nearly 30% of the membrane phosphatidylcholine, 20% of the phosphatidylethanolamine, 50% of the phosphatidylserine and 10% of the phosphatidylinositol (Wiegand & Anderson, 1983; Choe & Anderson, 1990). In frog ROS, phosphatidylethanolamine contains at least 50% dipolyunsaturated fatty acids followed by phosphatidylserine and phosphatidic acid. The most prevalent dipolyunsaturated fatty acids in phosphatidylserine and phosphatidylinositol are C22:6n-3 - C22:6n-3 along with various tetraenoic, pentaenoic and other polyenoic fatty acids, whereas phosphatidylcholine contained relatively more saturates and monoenes such as C16:0-C16:0, C16:0-C18:0, C16:0-18:0, C18:0-C18:1 and C22:6n-3 - C22:6n-3. The minor components of ROS, phosphatidic acid, phosphatidylinositol and phosphoglycerol, contain mostly C18:0-C20:4n-6 and C16:0-20:4n-6 (Stinson et al., 1991a). The peculiar feature of enriched dipolyunsaturated fatty acids could be correlated with the increase in the motionally restricted lipid component (Pontus & Delmelle, 1975). In classification of natural membrane (Daemen, 1973), ROS can be classified as a metabolically active type such as mitochondrial membrane which has a low cholesterol and sphingolipid content and a high amount of polyunsaturated fatty acids.

Lipid Metabolism

Studies of retina lipid composition and distribution imply that the retina offers an interesting model for studying retinal biosynthetic pathways in lipid metabolism. Until recently, the major emphasis in the study of retina lipid metabolism has been directed towards the elucidation of mechanisms for the biosynthesis of phospholipid and fatty acids for understanding the relationship between these membrane components and function of excitable membranes. Using several different isotopes, the biosynthetic route of lipid in the retina and ROS has been partially revealed in various ways *in vivo* and *in vitro* (Figure I-4).

De Novo Synthesis

Using radiolabeled glycerol as an index of *de novo* synthesis of lipid, studies both *in vivo* and *in vitro* have provided the metabolic pathway of phospholipid and glycerolipid in the retina (Bazan & Bazan, 1976; Giusto & Bazan, 1979a; Careaga & Bazan, 1981). When toad and bovine retina is incubated with radiolabeled glycerol, the time course of labeling sequence *in vitro* appeared to be phosphatidic acid, then diacylglycerol and finally triacylglycerol (PA-DG-TG pathway) (Giusto & Bazan, 1979a; Bazan & Bazan, 1976) (Figure I-4). The route of incorporation is through glycerol kinase and subsequent acylation (Giusto & Bazan, 1979a). High specific activities of glycerol are obtained in phosphatidic acid and phosphatidylinositol (PA-PI pathway) but

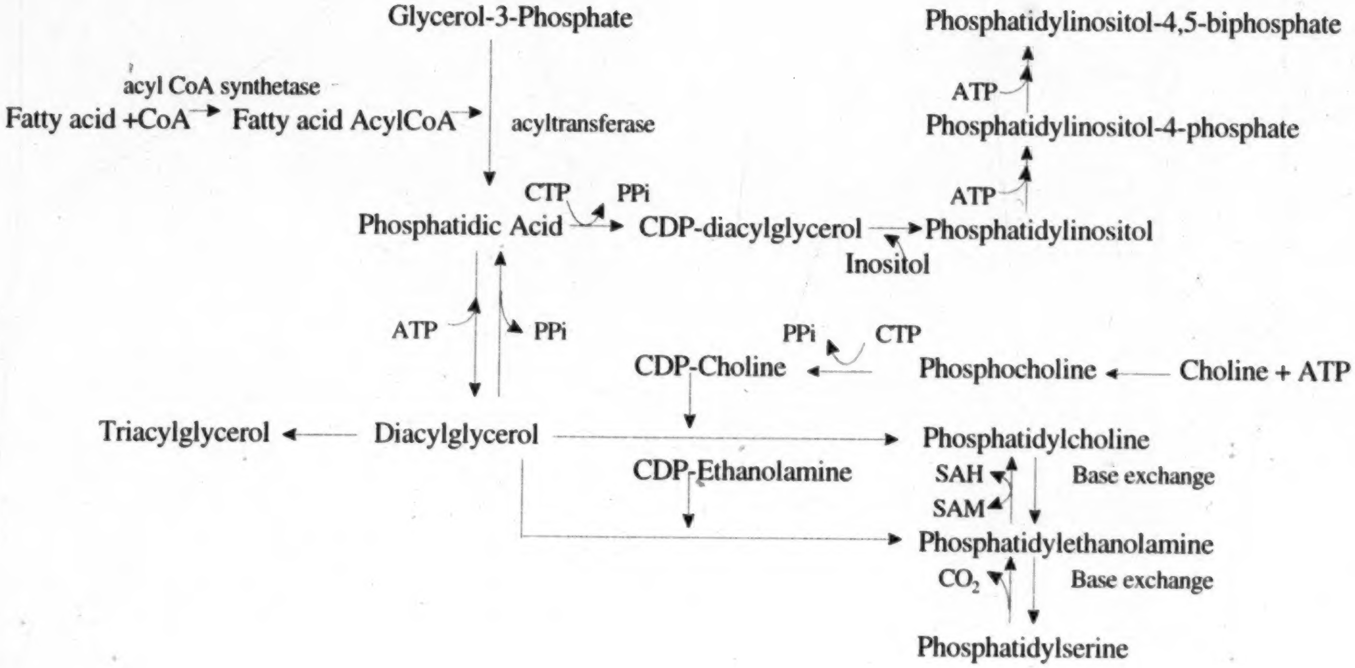
Glycerol-3-Phosphate
acyl CoA synthetase
Fatty acid +CoA
Fatty acid AcylCoA
acyltransferase
CTP
PPi
Phosphatidic Acid
CDP-diacylglycerol
Phosphatidylinositol
Inositol
ATP
Phosphatidylinositol-4-phosphate
ATP
Phosphatidylinositol-4,5-biphosphate
ATP
PPi
PPi
CTP
CDP-Choline
Phosphocholine
Choline + ATP
Triacylglycerol
Diacylglycerol
Phosphatidylcholine
CDP-Ethanolamine
SAH
SAM
Base exchange
Phosphatidylethanolamine
CO2
Base exchange
Phosphatidylserine

Figure I-4. Pathways of phospholipid synthesis.

decreased rapidly suggesting that the phosphatidate phosphohydrolase might be the rate limiting step in glycerolipid biosynthesis in the retina. Slow but steadily increasing labeling is obtained in phosphatidylcholine and phosphatidylethanolamine (PA-DG-PE/PC pathway) (Giusto & Bazan, 1979a; Bazan & Bazan, 1976; Wetzel & O'Brien, 1986). *In vitro*, a highly active [2-^{3}H]-glycerol uptake has also been found in phosphatidic acid of ROS prepared from rat, toad and bovine retina. *In vivo* labeling of [2-^{3}H]-glycerol in rat retina, phosphatidic acid and diacylglycerol displayed a rapid rate of synthesis and breakdown at an early time period (Careaga & Bazan, 1981), suggesting that active *de novo* biosynthesis of complex lipids takes place in the central nervous system (Bazan & Bazan, 1976). The highest rate of incorporation was attained for phosphatidylcholine followed by phosphatidylinositol which is the highest in microsomes. Diacylglycerol and phosphatidylethanolamine also take up 10 and 20% respectively of the precursor (Careaga & Bazan, 1981). These studies suggest glycerol labeling of lipids is accomplished by net biosynthesis rather than base exchange.

Several enzymes are involved in phospholipid biosynthesis. Swartz & Mitchell (1970) found the presence of *de novo* synthesis of phosphatidylcholine via Mg^{++} dependent CDP-choline pathway in rat retina by incubating retinal homogenate with radiolabelled phosphocholine and cytidine diphosphate choline (CDP-choline). This pathway is catalyzed by phosphocholine cytidyltransferase and phosphocholine transferase which were highest in microsomes and mitochondria, but are not active in ROS. CTP (Possmayer & Mudd, 1976), ATP (Erbland et al., 1967) and ions (Lamb & Fallon, 1974) are required for the biosynthesis of the major phospholipid through CDP-choline/ethanolamine phosphotransferase in several other tissues. Kennedy & Weiss (1956) observed the same route for phosphatidylcholine synthesis in the presence of diacylglycerol from phosphocholine in rat liver. Choline kinase activity was found in rabbit retina by Masland & Mills (1980). Labelled choline incorporated into phosphatidylcholine with high affinity. Dreyfus et al. (1978) also found phosphocholine/phosphoethanolamine phosphotransferase in chick and bovine retinal homogenate. These enzymes are active when retinal ROS are formed, but show different activity profiles during chicken retina ontogenesis, indicating two different enzymes as has been demonstrated in brain (Freysz et al., 1977).

Unlike whole retinal homogenate, phosphatidylinositol synthetase or phosphatidyl:cytidyl transferase which catalyze phosphatidylinositol formation from phosphatidic acid is not present in frog ROS. However, the ROS contains diacylglycerol, phosphatidylinositol-4,5-biphosphate (PIP_2) kinase activities for phophoinositides (Choe et al., 1990). Over a ten hour period, the rate of incorporation of labelled inositol and glycerol is the lowest among the various retinal fractions. The phosphatidylinositol molecules thus must be supplied by molecules derived from *de novo* synthesis in the inner segment of the photoreceptors (Choe et al., 1990), confirming that the inner segment photoreceptor is a major site of phospholipid synthesis.

Base Exchange

An alternative way for biosynthesis of phospholipid by base exchange has been reported in nervous tissue (Porcellati et al., 1971; Mizuno, 1976). This enzymatic system is able to change the hydrophilic head of phosphoglyceride molecules by exchanging, without using ATP, its nitrogenous base with ethanolamine, choline or serine present in the cells as free bases (Gaiti et al., 1986). Mizuno (1976) found that retina has a higher rate of Ca^{2+}-dependent serine and ethanolamine incorporation into phospholipid with the highest activity in microsomes from rabbit retina, while the ROS had very little activity. This confirms that phospholipid is synthesized in the inner segment microsomes using CDP-based derivatives and then transferred to the ROS as suggested by Young (1976). However, in the absence of an alternative pathway for phosphatidylserine synthesis which incorporates labelled glycerol into phosphatidylserine in canine retina *in vivo* (Wetzel et al., 1989), base exchange may be the only major source of retinal phosphatidylserine (Fliesler & Anderson, 1983). Illumination does not affect base exchange of ethanolamine and serine in retina or ROS, thus it may not play a direct role in the visual process (Mizuno, 1976).

Decarboxylation and Transmethylation

Decarboxylation of phosphatidylserine is one of the sources for phosphatidylethanolamine biosynthesis which is also derived from *de novo* synthesis and base exchange. All three pathways have been demonstrated in the retina (Anderson et al., 1980a & 1980b; Mizuno, 1976; Dreyfus, 1978). Two pathways have been discussed earlier. The frog retina incubated with [^{3}H]-serine actively labeled into phosphatidylethanolamine via decarboxylation of phosphatidylserine in ROS and microsomes (Anderson et al., 1980b). Aveldano et al. (1983) also found that bovine retinal microsome incorporated labelled serine into both phosphatidylserine and phosphatidylethanolamine. Since labelled glycerol is not actively incorporated into retina phosphatidylethanolamine (Careaga & Bazan, 1981) nor is ethanolamine an effective precursor of phosphatidylethanolamine (Anderson et al., 1980a), the major pathway for phosphatidylethanolamine synthesis in the retina is via the decarboxylation reaction (Anderson et al., 1980a).

Transmethylation is another pathway for phosphatidylcholine synthesis by stepwise methylation of phosphatidylethanolamine (Bremer et al., 1960). This pathway has been observed in retinal microsomes and ROS (Anderson et al., 1980c). Phosphatidylethanolamine derived via *de novo* biosynthesis from labeled ethanolamine or base exchange are active substrates for this pathway, whereas phosphatidylethanolamine derived via decarboxylation of phosphatidylserine is poorly methylated (Anderson et al., 1980a & 1980c; Fliesler & Anderson, 1983), although the reason is not clearly addressed. The physiological role of transmethylation in photoreceptor cells remains unclear, but the function may be related with vesicle fusion that is part of the process in new membrane assembly since the reaction is particularly active in basal disc of the ROS (Fliesler & Anderson, 1983).

Acyl Group Determination

The incorporation of polyunsaturated fatty acids into phospholipid plays a key role in determining characteristic patterns of molecular species of phospholipids. It provides a suitable microenvironment for proper membrane function. Two major mechanisms of fatty acid incorporation into phospholipid molecules in the retina involve *de novo* synthesis and reacylation. Activation of a fatty acid to its coenzyme A (CoA) derivatives via ATP, CoA, $MgCl_2$ dependent acyl CoA synthetase must occur before further fatty acid metabolism occurs (Groot et al., 1976) (Figure I-3). The long chain acyl CoA synthetases are localized primarily in the microsomal fraction of the retina, pigment epithelium (Reddy & Bazan, 1985) and photoreceptor membrane (Zimmerman & Keys, 1986; Giusto et al., 1986). Reddy and coworkers (1984) suggest the possible existence of a single long chain acyl CoA synthetase with different affinity for fatty acids in brain tissue. The enzyme has a higher affinity for docosahexaenoic acid in retina than in pigment epithelium (Reddy & Bazan, 1985). The activity of long chain acyl coenzyme A synthetase in homogenate and microsomes of retina with various fatty acids showed the following pattern of incorporation: arachidonic acid > linoleic acid > palmitic acid > docosahexaenoic acid (Reddy & Bazan, 1984). Although the docosahexaenoyl CoA synthetase activity is low, its affinity towards C22:6n-3 is higher compared to C20:4n-6 and C16:0 in brain (Reddy & Bazan, 1985) and retina (Reddy & Bazan, 1984). The synthesis of acyl CoA thus may play a central role in the retention of polyunsaturated fatty acids within cells, particularly the retina.

Activated fatty acyl CoA can then incorporate into phospholipid via acyltransferase through *de novo* synthesis of phosphatidic acid in retinal microsomes (Bazan et al., 1982a & 1982c). Under optimal conditions in the presence of exogenous lysophosphatidic acid as acceptors, most of the $[^{14}C]$-22:6n-3 incorporated into phosphatidic acid. The docosahexaenoic acid containing phosphatidic acid is further metabolized to diacylglycerol, triacylglycerol, phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine. Phosphatidylethanolamine has the highest level of incorporation (Bazan et al., 1986a). In bovine retinal microsomes, phosphatidic acid contains 21% of the total esterified docosahexaenoic acid (Giusto & Bazan, 1979b) and 65% of acyl group of phosphatidic acid is composed of docosahexaenoic acid (Bazan et al., 1982a & 1982c). Thus, this pathway might be responsible for synthesis of a large proportion of the highly unsaturated molecular species of membrane lipids.

Phospholipid species produced *de novo*, such as docosahexaenoic acid containing phospholipids, would then be modified to give specific molecular species through interaction with other enzymatic reacylation-deacylation systems. The presence of an acyltransferse having various degrees of substrate preference and positional specificity in bovine retinal microsomes and ROS (Swartz & Mitchell, 1974; Zimmerman & Keys, 1988 & 1989; Giusto et al., 1986), phospholipase A, and lysophopholipase in retinal micorosomes, whole homogenate, ROS and pigment epithelium (Swartz & Mitchell, 1973; Zimmerman & Keys, 1988 & 1989) has been reported. With this enzyme system, the acylation-deacylation could lead to independent turnover of the fatty acid esters of phospholipid molecules without extensive degradation and *de novo* synthesis (Lands, 1960). When radiolabelled C22:6n-3 is incubated with retinal microsomes with lysophosphatidylcholine, acyltransferase introduced docosahexaenoic acid into phosphatidylcholine with no increase in incorporation into diacylglycerol (Bazan & Bazan, 1985). Acylation of arachidonic acid is mainly into phosphatidylinositol (Wetzel & O'Brien, 1986). Substrate preferences for fatty acyl CoA incorporation into phosphatidylcholine using lysophosphatidylcholine (1-palmitoyl-*sn*-glycero-phosphocholine) as an acceptor are C18:0>C20:4n-6>C18:2n-6≥C22:6n-3 and for phosphatidylethanolamine using lysophosphatidylethanolamine (1-palmitoly-*sn*-glycerophosphoethanolamine) are C20:4n-6=C22:6n-3>C18:0>C18:2n-6. Polyunsaturated fatty acyl CoA is thus favoured for incorporation into phosphatidylethanolamine and to a lesser extent into phosphatidylcholine (Sellner & Phillips, 1991), consistent with fatty acid composition data.

It has also been reported that this acylation-deacylation system is active in ROS to give selective fatty acid composition (Louie et al., 1991; Giusto et al., 1986; Wetzel et al., 1989). About 80% of labelled docosahexaenoic acid appears in phosphatidylcholine of ROS and two-thirds of the label is in its dipolyunsaturated fatty acid molecules and one-third in hexaene (Giusto, 1986) through this system. Arachidonic acid is primarily labelled in phosphatidylinositol and phosphatidylcholine (Wetzel & O'Brien, 1986).

Elongation and Desaturation

The existence of an elongation-desaturation system in retina (Bazan et al., 1982b) and ROS (Wetzel et al., 1991) has been reported. *In vivo* after intravitreal injection of [1-^{14}C]22:5n-3, more than 30% of the precursor is converted into C22:6n-3 (Bazan et al., 1982b). The highest and most rapid labelling in retina is found in phosphatidylcholine, phosphatidylinositol and phosphatidic acid. These results indicate that elongation and desaturation is operative during phosphatidic acid synthesis and supports *de novo* synthesis of polyenoic fatty acids of phospholipids. A substantial conversion of [^{14}C]18:3n-3 to labelled C20:5n-3, C22:5n-3 and C22:6n-3 was noted in ROS with a subsequent increase in C22:6n-3 over the period (Wetzel et al., 1991). The conversion of C22:5n-3 to C22:6n-3 by Δ^4 desaturase which is very active in chick brain and retina (Anderson et al., 1990) appears to be rate limiting and may thus affect phospholipid replacement during photoreceptor outer segment renewal (Wetzel et al., 1991). However, the existence of Δ^4 desaturase is questioned by Voss et al. (1991 & 1992), although the finding of these authors has not been identified in retina or brain.

These data from activation-acylation by *de novo* synthesis, activation-acylation-deacylation system, and desaturation-elongation indicate the means by which the high level of polyunsaturated fatty acids, especially docosahexaenoic acid containing phospholipids, is retained in retina and photoreceptor cells.

B. VERY LONG CHAIN FATTY ACIDS

Long chain polyenoic fatty acids with less than 22 carbons in chain length have been the main target of many studies. Over the last few years, attention has focused on another important membrane component termed very long chain fatty acids with carbon chain lengths greater than 22. These fatty acids are enriched in specialized tissues such as retina, brain, sperm and testes and in peroxisomal diseases. The peroxisome is the main organelle responsible for β-oxidation of very long chain fatty acids. The accumulation of very long chain fatty acid is found in brain and skin fibroblasts of patient with the absence or abnormality of peroxisomes. Information concerning the metabolic synthesis and function of very long chain fatty acids is limited and remains to be elucidated.

Structure and Tissue Distribution

Very long chain fatty acids occur in vertebrate tissues (Table I-4): human and rat brain (Poulos et al., 1988; Robinson et al., 1990a), vertebrate retina (Aveldano, 1987 & 1988; Aveldano & Sprecher, 1987), rat testis (Bridges & Coniglio, 1970; Grogan, 1984), mouse spermatocytes and spermatids (Grogan & Huth, 1983), mammalian spermatozoa (Poulos et al., 1986c; Robinson et al., 1992), human vascular endothelial cells (Rosenthal & Hill, 1984) and human skin fibroblasts (Street et al., 1989). High levels of these fatty acids are also found in human brain with inherited peroxisomal disease such as Zellweger's syndrome and adrenoleukodystrophy (Poulos et al., 1988; Poulos et al., 1986a).

In both brain and retina, very long chain fatty acids are polyenoic with 4, 5, or 6 double bonds and belong to n-6 and n-3 families with carbon chain lengths up to 36 in retina (Aveldano, 1987) or 40 in brain (Poulos et al., 1986b; Sharp et al., 1991). These fatty acids occur exclusively in phosphatidylcholine and are esterified mainly in the *sn*-1

Table I-4. Very long chain (C24-C40) polyenoic fatty acids in vertebrate tissues.

Tissue	Vertebrate	Phospholipid	Series	Double bond	Carbon atom (maximum chain length)	Reference
Brain	Human (normal)	Phosphatidylcholine	n-6	4 5 6	38	Poulos et al. (1988)
	Human (Zellweger's)	Phosphatidylcholine Cholesterol ester	n-6	5 6	40	Poulos et al. (1986a) Sharp et al. (1987 & 1991)
	Rat	Phosphatidylcholine	n-6 n-3	4 5 6	38	Robinson et al. (1990a)
Retina	Bovine, Rabbit, Rat, chicken	Phosphatidylcholine	n-6 n-3	4 5 6	36	Aveldano (1987)
	Cod	Phosphatidylcholine	n-3	5 6	32	Aveldano (1987)
Testes & Spermatozoa	Rat*	--	n-6	4 5	30**	Grogan (1984)
	Ram Bull	Sphingomyelin	n-6 n-3	4 5 6	34	Robinson et al. (1992) & Poulos et al. (1986c)
	Rat, Boar	Sphingomyelin	n-6	3 4 5	34	"
	Human	Sphingomyelin	n-6	2 3 4	32	"
Vascular endothelial cells	Human	--	n-6	4 5	26***	Rosenthal & Hill (1984)

*, testes alone. **, fatty acids synthesized from arachidonic acid (C20:4n-6). ***, fatty acids synthesized from eicosatrienoic acid (C20:3n-6) in culture.

position of the glycerol backbone, whereas saturated, monounsaturated and other polyunsaturated fatty acids are present at the *sn*-2 position (Aveldano, 1988; Poulos et al., 1988; Robinson et al., 1990a). This positioning contrasts to that typical of most membrane phosphatidylcholine species in other tissues.

In retina, the polyenoic very long chain fatty acids occur exclusively in dipolyunsaturated phosphatidylcholine and account for 2 mol% and 13 mol% of phosphatidylcholine in retina and ROS, respectively (Aveldano, 1987). The major phosphatidylcholine species of bovine retina are decaenes; C30:4n-6-C22:6n-3, C32:4n-6-C22:6n-3 and C34:4n-6-C22:6n-3, undecaenes; C30:5n-3-C22:6n-3, C32:5n-3-C22:6n-3 and C34:5n-3-C22:6n-3 and dodecaenes; C30:6n-3-C22:6n-3, C32:6n-3-C22:6n-3 and C34:6n-3-C22:6n-3 (Aveldano & Sprecher, 1987). The levels of these fatty acids are highly enriched in ROS. The level of C32:5n-3, C32:6n-3, C34:5n-3 and C34:6n-3 in ROS is even higher than for 20 and 22 carbon polyenes with exception of docosahexaenoic acid. Polyenoic very long chain fatty acids with 24 and 26 carbons are more prevalent constituents in phosphatidylserine than in phosphatidylcholine (Aveldano, 1987).

Aveldano (1987) also found that different animal species have different very long chain fatty acid patterns in retina. Cod (*Gadus morrhua*) retina contains the largest amount of very long chain fatty acids in phosphatidylcholine (C22:6n-3 and C32:6n-3, 65% and 15%, respectively), whereas the smallest amount is found in toad retina. Chicken and rabbit retina contain predominantly n-6 pentaenes, C22:5n-6 to C36:5n-6. Rat contains the highest proportion of n-3 hexaenes in the retina (Aveldano, 1987). These results imply that the fatty acid composition of each species in retina may reflect diet and diversified visual cell structures between species. Thus, it may be possible to modulate the retinal fatty acid compositions by feeding different sources of dietary fat.

In normal human brain, the polyenoic very long chain fatty acids represent a minor component of the total fatty acid fraction but is increased in brain with Zellweger's syndrome, an inherited disease characterized by peroxisomal deficiency (Poulos et al., 1986a, 1986b, & 1988). The fatty acid composition is also different in normal and diseased brain. Tetra- and pentaenoic n-6 derivatives are the major 32-38 carbon fatty acids in normal brain, whereas Zellweger patients contain predominantly pentaenoic and hexaenoic acids (Poulos et al., 1988; Sharp et al., 1991). In normal rat brain, these fatty acids belong to both n-3 and n-6 series up to 38 carbons (Robinson et al., 1990a). Johnson et al. (1992) reported the existence of very long chain monoenoic fatty acids in human brain up to 28 carbons in chain length. These fatty acids contain predominantly two positional isomer series, the n-7 and n-9 *cis* homologues. Robinson et al. (1990a) speculates that this unique brain lipid may be required for the correct orientation of intrinsic membrane proteins.

In testes and spermatozoa, polyenoic very long chain fatty acids occur almost exclusively in sphingomyelin and ceramide (Robinson et al., 1992). These fatty acids represent approximately 15% of the total sphingomyelin fatty acids in testes and spermatozoa of adult animals and account for approximately 5% of the total fatty acids in spermatozoa (Poulos et al., 1986c; Robinson et al., 1992). The fatty acid composition varies markedly according to species (Poulos et al., 1986c). In human spermatozoa it belongs to only the n-6 series up to 32 carbons, but rat and boar testes and spermatozoa contain mainly n-3 fatty acids with up to 34 carbons in chain length. Testes and spermatozoa of ram and bull contain both n-6 and n-3 series with 4, 5 and 6 double bonds of up to 34 carbons. Rat and boar testes and spermatozoa also contain a substantial amount of α-hydroxyl-polyenoic very long chain fatty acids in sphingomyelin and ceramide (Robinson et al., 1992). Along with the high content of C22:6n-3, C22:5n-6 and C20:4n-6 (65%) in testes and spermatozoa (Poulos et al., 1973) which apparently have effects upon spermatogenesis (Marzouki & Coniglio 1982), the occurrence of these polyenoic fatty acids may play a crucial role in maintaining membrane integrity of spermatozoa.

Biological function

The physiological role of the polyenoic very long chain fatty is essentially not known. Aveldano (1987 & 1988) speculated that these fatty acids may be necessary to make photoreceptor proteins, rhodopsin. After hexane extraction of lysophilized discs, phosphatidylcholine is the major phospholipid remaining with rhodopsin, followed by phosphatidylserine. This rhodopsin bound lipid has more polyenoic long chain and very long chain fatty acid (Aveldano, 1988) compared to hexane extracted lipid, implying that very long chain fatty acids at the sn-1 position of the phospholipid may have a high affinity to bond with protein. Recently, the possible involvement of very long chain fatty acid in membrane signal transduction has been explored in rat brain and human neutrophils. C32:4n-6 and C34:6n-3 activate purified rat brain protein kinase C (PKC) *in vitro* with maximal activity being between 25 and 50 μM (Hardy et al., 1994). The authors also observed the same effect when long chain fatty acid (C20:4n-6, C20:5n-3 or C22:6n-3) is used. Therefore it is hard to distinguish the unique role of very long chain fatty acids with this result. With a subsequent experiment, the same authors (1995) report that tetraenoic very long chain fatty acid induce Ca^{2+} mobilization in human neutrophils, which involve both the release of Ca^{2+} from the intracellular stores and changes to the influx or efflux of the ion. They also observe that C30:4n-6 mobilize Ca^{2+} from a thapsigargin-insensitive intracellular pool. Superoxide production induced by very long chain fatty acids declined progressively from thapsigargin pretreatment. However, both Ca^{2+} mobilization and superoxide production induced by C20:4n-6 are inhibited by thapsigargin. Thus the authors (1995) conclude that biological activity for the very long chain fatty acid is distinct from long chain fatty acid in influencing second messenger systems in intact cells. The question arise whether the above results can be seen in other tissues containing very long chain fatty acids, since neutrophils contain the fatty acid only up to C28 and $[1\text{-}C^{14}]$30:4n-6 is poorly metabolized in these cells (Robinson et al., 1994).

Developmental Changes

The function of polyenoic very long chain fatty acids both in phosphatidylcholine and sphingomyelin may be related with a particular stage of development since both their concentration and composition change markedly with age in brain retina and testes (Robinson et al., 1990a; Sharp et al., 1991; Robinson et al., 1992). In the neonatal rat (1 day old) and human fetal brain of 24 week gestation, the predominant very long chain polyenoic fatty acids are n-6 pentaenes: C25:5, C32:5 and C34:5. In contrast, tetraenes such as C24:4, C34:4 and C36:4 are the major very long chain fatty acids present beyond the neonatal period (Robinson et al., 1990b; Sharp et al., 1991), indicating that n-6 pentaenes of very long chain fatty acids dramatically decrease with maturation. During gestation and early infancy, monoenoic very long chain fatty acid content in phosphatidylcholine is negligible, however, this increases rapidly to a maximum at about two years of age in parallel with the process of myelination (Sharp et al., 1991). For example, C26:0, C24:1, C26:1n-9 and C26:1n-7 do not appear in human brain tissue until postnatal life and increase rapidly as myelination progresses (Martinez, 1991). Peroxisomal activity is increased with the onset of myelination and enzymes for very long chain fatty acid catabolism are also active (Lazo et al., 1991). This indicates peroxisomal involvement in myelin assembly and turnover. The level of C22:6n-3 and very long chain fatty acids in dipolyunsaturated fatty acids in phosphatidylcholine in retina decrease with age (Rotstein et al., 1987). The polyenoic very long chain fatty acids in sphingomyelin of rat testes vary from birth to sexual maturity (Robinson et al., 1992). These fatty acids are not present in the infant until 25 days after birth. Between 35 to 50 days after birth all very long chain fatty acids up to 32 carbons can be detected and increase in total amount during this period (Robinson et al., 1992). This indicates that very long chain polyenoic fatty acids are associated with spermatid development and cell differentiation.

Biosynthesis of Very Long Chain Fatty Acids

Many studies have found and confirmed that very long chain polyenoic fatty acids are synthesized by successive elongation from shorter chain precursors in retina (Rotstein & Aveldano, 1988), brain (Robinson et al., 1990b) and testes (Grogan, 1984; Grogan & Huth, 1983). Human vascular endothelial cells also synthesize and release C24:4n-6, C24:5n-6, C26:4n-6 and C26:5n-6 in culture by elongation and desaturation from their precursors C20:3n-6, C20:4n-6 and C22:4n-6 (Rosenthal & Hill, 1984).

Since phosphatidylcholine in retina, containing very long chain polyenoic fatty acid, is the most highly labeled product from [^{14}C]acetate incubation *in situ*, Rotstein & Aveldano (1988) speculate that phosphatidylcholine could provide acyl CoA polyenes which are a substrate for an elongase system. Such acyl CoAs may be synthesized either from free fatty acids and CoA or from phospholipids by an ATP independent acyltransferase catalyzed reaction: CoA + PL ↔ acyl CoA + lysoPL (Giusto et al., 1986). Elongated acyl CoA could then be reacylated into phosphatidylcholine for synthesis of very long chain fatty acids. At present, it is not known whether the elongase and desaturase for the synthesis of polyunsaturated fatty acids with less than 24 carbons are involved in the synthesis of the polyenoic very long chain fatty acids or whether there is a separate elongase or individual elongases specific for the n-3 and n-6 series (Poulos et al., 1986). In addition to the biosynthesis, Rotstein et al. (1996) suggest that actively formed C24:5n-3 from [^{14}C]22:5n-3 in retina maybe an important precursor for longer pentaenes via elongation. However, other possible candidates for the precursor have not been tested.

Peroxisomal Metabolism

Peroxisomal β-oxidation System

A peroxisome is 0.5 μm in diameter surrounded by a single membrane and filled with a fine granular matrix (Rhodin, 1954). De Duve et al. (1960) characterized it as a biochemically distinct entity and later Lazarow & De Duve (1976) emphasized its important role in fatty acid β-oxidation. Since then both peroxisomes and mitochondria are known to be involved in β-oxidation by chain shortening. Peroxisomal β-oxidation, like mitochondrial β-oxidation, proceeds via successive steps of dehydrogenation, hydration, dehydrogenation and thiolytic cleavage. However, the enzyme system of the peroxisome is distinct from the mitochondrial system (Hashimoto, 1982) (Figure I-5). The first step of peroxisomal β-oxidation is acyl CoA oxidase, yielding H_2O_2 (Lazarow & De Duve, 1976). It is not reactive with short chain acyl CoA esters and thus β-oxidation is not likely completed in peroxisomes (Lazarow, 1978). Two reactions are catalyzed by bifunctional proteins: enoyl CoA hydratase and L-3-hydroxyacyl CoA dehydrogenase (Furuta et al., 1980). Finally, the last reaction in peroxisomal β-oxidation

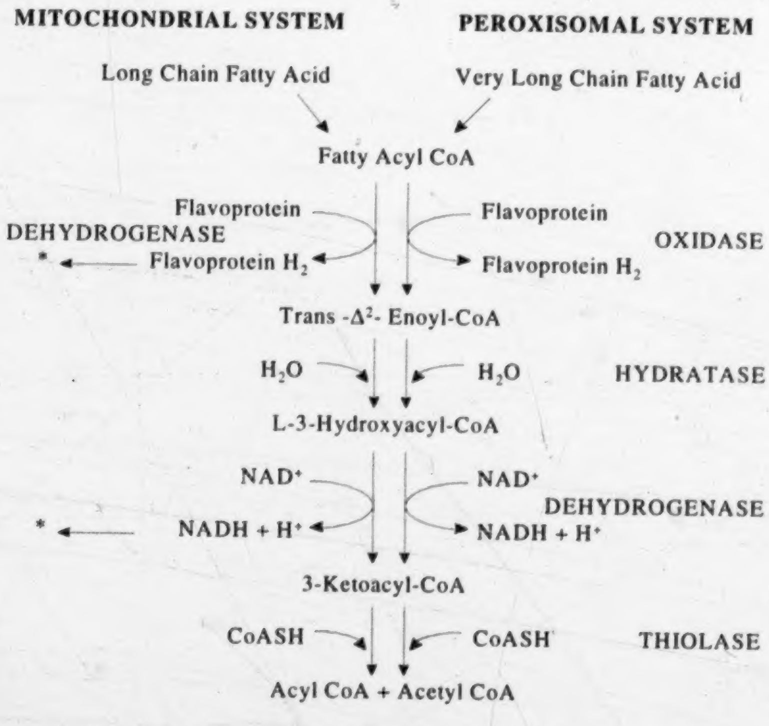
MITOCHONDRIAL SYSTEM
PEROXISOMAL SYSTEM
Long Chain Fatty Acid
Very Long Chain Fatty Acid
Fatty Acyl CoA
Flavoprotein
DEHYDROGENASE
* Flavoprotein H2
Flavoprotein
OXIDASE
Flavoprotein H2
Trans -Δ2- Enoyl-CoA
H2O
H2O
HYDRATASE
L-3-Hydroxyacyl-CoA
NAD+
NAD+
DEHYDROGENASE
* NADH + H+
NADH + H+
3-Ketoacyl-CoA
CoASH
CoASH
THIOLASE
Acyl CoA + Acetyl CoA

Figure I-5. β-Oxidation of fatty acids in mitochondria and peroxisome. * products go to respiratory chain.

is catalyzed by peroxisomal 3-ketoacyl CoA thiolase (Miyazawa et al., 1981). This enzyme has no activity with acetoacetyl CoA but is highly active with longer chain substrates (Miyazawa et al., 1981). The exact function of peroxisomal metabolism is not clear, the current studies exhibits the involvement of peroxisome in the initial steps of very long chain fatty acid oxidation.

Saturated and Monounsaturated Very Long Chain Fatty Acids

Kawamura et al. (1981) first observed that a saturated very long chain fatty acid, lignoceric acid (C24:0), is oxidized in peroxisomes. Subsequently, Singh et al. (1984) confirmed that the major part of β-oxidation of very long chain fatty acids takes place in peroxisomes in rat liver and human fibroblasts. Peroxisomal oxidation is not inhibited by potassium cyanide (KCN) which depressed palmitate β-oxidation drastically (Kawamura, 1981; Singh et al., 1984) and is almost completely dependent on ATP, coenzyme A, Mg^{2+} and NAD^+ (Wanders et al., 1987b). The activation of lignoceric acid and cerotic acid (C26:0) via CoA synthetase occurs in the endoplasmic reticulum and peroxisomes but not in mitochondria (Wanders et al., 1987b; Lageweg et al., 1991; Singh & Poulos, 1988) and these CoA synthetases are located in the cytosolic face of the peroxisomal membrane (Lageweg et al., 1991). These enzymes are deficient in peroxisomal diseases such as Zellweger's syndrome (Wanders et al., 1987a), confirming them as peroxisomal enzymes. After activation to CoA derivatives, very long chain fatty acids are degraded by both mitochondria and peroxisomes (Singh & Poulos, 1988), suggesting that a specific very long chain fatty acid CoA synthetase may be required for activation of the free fatty acid and may regulate the β-oxidation of very long chain fatty acids in the cell (Singh et al., 1987).

Dietary fat has been involved in induction of peroxisomal β-oxidation (Flatmark et al., 1988; Bremer & Norum, 1982). Twenty percent of hydrogenated fish oil in a semipurified diet increases peroxisomal β-oxidation in rat liver (Flatmark et al., 1988).

Continued feeding of diets with C22:1 also increase β-oxidation enzyme systems in peroxisomes (Bremer & Norum, 1982).

Polyenoic Long and Very Long Chain Fatty Acids

Both mitochondria and peroxisomes contain the enzymatic equipment to catalyze β-oxidation of unsaturated fatty acids (Dommes et al., 1981). Degradation of unsaturated fatty acids requires the participation of 2,4-dienoyl-CoA reductase and Δ^3-*cis*-Δ^3-*trans*-enoyl-CoA isomerase (Schulz & Kunau, 1987). These enzymes are in both mitochondria and peroxisomes as two independent enzymes (Dommes et al., 1981), implying that peroxisomes have the capacity for polyunsaturated fatty acid β-oxidation. Hovik & Osmunsen (1987) found that the peroxisomal β-oxidation system shows preference towards long chain polyunsaturated fatty acids. Recently, β-oxidation of C24:4n-6 (tetracosatetraenoic acid), C20:4n-6 and C24:0 in peroxisomes of cultured skin fibroblasts has been reported (Street et al., 1989 & 1990; Moore et al., 1995). The main products of β-oxidation are CO_2, C14-C24 saturated and monounsaturated fatty acids formed from released acetate and water soluble product. All of these products are decreased but elongation is above normal in skin fibroblasts in Zellweger's syndrome as well as in other peroxisomal diseases, implying that the elongation is not a peroxisomal system. These results indicate that one of the major peroxisomal functions is the generation of a two carbon unit for synthesis of fatty acids such as palmitic and stearic acids and that function is impaired in Zellweger's syndrome (Street et al., 1989). Fatty acid elongation also plays an important role in the accumulation of very long chain fatty acids in cells (Koike et al., 1991).

Retroconversion

The idea of retroconversion is accepted but not a thoroughly tested hypothesis (Sprecher, 1991). The retroconversion mechanism has been reported in mitochondria

(Stoffel et al., 1970). However, recently Gronn et al. (1990 & 1991) reported peroxisomal retroconversion of C22:6n-3 to C20:5n-3 in rat liver. Twenty percent is retroconverted by one cycle of β-oxidation via Δ^4-enoyl CoA reductase and Δ^3,Δ^2-enoyl CoA isomerase. This conversion is not inhibited by either carnitine which stimulates mitochondrial β-oxidation or by decanoylcarnitine which inhibits mitochondrial β-oxidation (Gronn et al., 1991). Retroconverted C20:5n-3 is rapidly incorporated into triacylglycerol, phosphatidylcholine and phosphatidylethanolamine (Gronn et al., 1991). This retroconversion is deficient in fibroblast cultures from Zellweger's syndrome (Gronn et al., 1990) suggesting that the reaction is a peroxisomal function. This peroxisomal retroconversion was positively tested by Voss et al. (1991 & 1992), demonstrating that the formation of C22:6n-3 occurs in liver via elongation of C22:5n-3 to C24:5n-3 and desaturation to C24:6n-3 at Δ^6 position and then by chain shortening to C22:6n-3. Since then the peroxisomal retroconversion has been confirmed in humans and rats (Brossard et al., 1996) and in human skin fibroblasts (Moore et al., 1995; Spector et al., 1997). Metabolites produced by peroxisomal β-oxidation can be further oxidized or move back to the microsome for elongation. This implies that the corporation between peroxisomes and microsomes is important in unsaturated fatty acid biosynthesis. Marzo et al. (1996) suggest that two different Δ^6-desaturase activities may exist. One would correspond to the classical Δ^6-desaturase, responsible for the conversion of C18:2n-6 to C18:3n-6 and its counterpart of the n-3 fatty acid family. The other enzyme could utilize C24:4n-6 and C24:5n-3 as substrates. Together with the data of Voss et al. (1991), this implies that the latter enzyme may be located in the peroxisome.

From studies of the fatty acid profile of Zellweger's syndrome, Martinez (1990 & 1991) found striking changes in polyunsaturated fatty acid composition. There are significant decreases in Δ^4 desaturase products, C22:6n-3 and C22:5n-3. Docosahexaenoic acid is virtually negligible in liver from Zellweger syndrome patients. There is a complementary increase in C18:2n-6 and C20:3n-6 (Martinez, 1991). The ratios in Zellweger's syndrome of C26:0/C22:6n-3 and C26:1/C22:6n-3 are almost 150 times higher than normal values which may make these ratios indices of Zellweger's syndrome. Thus, Martinez (1989) has suggested that Δ^4 desaturase could be one of many peroxisomal enzymes and that the deficiency of docosahexaenoic acid synthesis could explain much of the neurological and visual symptoms in peroxisomal disease. In view of Sprecher's new pathway (Voss et al., 1991 & 1992), the existence of Δ^4 desaturase in peroxisome is doubtful.

Human Disease of Very Long Chain Fatty Acids

Peroxisomal Disease

Zellweger's syndrome (cerebro-hepato-renal syndrome) and adrenoleukodystrophy (ALD, x-linked or neonatal) are inherited human metabolic diseases. They are characterized by either the absence of (Zellweger's syndrome) or abnormalities (ALD) of peroxisomes (Goldfischer et al., 1973). There is an impairment of peroxisomal β-oxidation with toxic accumulations of very long chain fatty acids which cause neurological abnormalities and death in both cases. The most characteristic clinical findings in Zellweger's syndrome are hypotonia, severe mental retardation, seizures and an atypical face. Ninety percent of cases die during infancy (Brown et al., 1982; Kelley, 1983). The clinical symptoms in adrenoleukodystrophy overlap with Zellweger's syndrome but predominately affects 5-15 year old males with demyelination of the central nervous system especially in the cerebral white matter (Brown et al., 1982). Saturated and monounsaturated very long chain fatty acids such as C24:0, C24:1, C26:0 and C26:1 are accumulated in liver or skin fibroblasts (Moser et al., 1984b; Poulos et al., 1986a). Polyenoic very long chain fatty acids up to 38 carbons in chain length accumulated in plasma and brain of Zellweger's syndrome patients (Poulos et al., 1986a & 1986b). Severe depletion of docosahexaenoic acid levels in Zellweger's syndrome has also been found (Martinez, 1991; Martinez et al., 1994). Arachidonic acid is decreased in plasma but normal in erythrocyte with increased level of linoleic acid (Martinez et al., 1994). These accumulations are the result of defects in peroxisomes and deficient enzyme function. Very long chain fatty acyl CoA synthase (Wanders et al, 1990; Singh & Poulos, 1988), enoyl CoA hydratase and L-3-hydroxyacyl CoA dehydrogenase (Chen et al., 1987) are deficient in adrenoleukodystrophy and all enzymes for β-oxidation are absent in liver from Zellweger's syndrome patients (Chen et al., 1987; Singh et al., 1987). Recently, the putative gene for ALD, adrenoleukodystrophy protein (ALDP), has been identified (Mosser et al., 1993). This protein has an ATP-binding region and belongs to the ATP-binding cassette (ABC) transporter protein superfamily which are involved in transport of proteins, amino acid, and inorganic ions. Thus, the transport of very long chain fatty acyl CoA synthase or very long chain fatty acid into the peroxisomal matrix may be impaired in adrenoleukodystrophy (Valle & Gartner, 1993). Very recently, Kobayashi et al. (1997) generated a line of mice deficient in ALDP which exhibits a significant increase in C26:0 with no sign of demyelination in brain and spinal cord. Together with finding ALDP, this animal model may enlighten the pathogenesis of peroxisomal disease.

Diet Therapy

There is evidence that the accumulated very long chain fatty acids are at least partly from dietary origin (Kishimoto et al., 1980). In x-linked adrenoleukodystrophy, dietary restriction of very long chain fatty acids (C26:0) with glycerol trioleate oil feeding has significantly lowered saturated very long chain saturated fatty acid levels in plasma (Van Duyn et al., 1984; Moser et al., 1984a; Rizzo et al., 1987). However, this is accompanied by an increase in monoenoic very long chain fatty acids, therefore the level of very long chain fatty acid is unchanged. A patient with Zellweger syndrome fed glycerol trioleate oil milk formula, resulted in a decrease of about 50% of plasma very long chain fatty acids within 1 month but does not slow clinical deterioration (Tylki-Szymanka & Stradomska, 1995). The new trial with glycerol trierucate ("Lorenzo's oil") with glycerol trioleate reduce further the synthesis of very long chain fatty acid within four to six weeks (Rizzo et al., 1989). However this dietary trial does not provide evidence of a clinically relevant benefit in patients with adrenoleukodystrophy (Rizzo et al., 1989) and adrenomyeloneuropathy (Aubourg et al., 1993). One of the disappointing reasons is that erucic acid is unable to cross the blood brain barrier (Poulos et al., 1994; Rasmussen et al., 1994). Therefore the endogeneous synthesis of saturated VLCFA is not reduced in brain. An additional side effect of feeding erucic acid is the reduction of platelet count (Kickler et al., 1996) and docosahexaenoic acid (Poulos et al., 1994). More recently docosahexaenoic acid therapy applied in patients with Zellweger syndrome was reported to give more favorable clinical changes in addition to improved erythrocyte lipid profiles, visual improvement, better visual and social contact and improved muscle tone (Martinez, 1996). All the above dietary trials are applied in patients who already show the characteristic symptoms of the disease. Thus it would be interesting to see the results when the nutritional therapy starts earlier when the patients are free of symptoms.

C. FACTORS AFFECTING RETINAL FATTY ACID COMPOSITION AND FUNCTION

It is now clear that n-6 and n-3 polyenoic long and very long chain fatty acids are major components of photoreceptors in the retina and central nervous system. Particularly docosahexaenoic acid and its longer elongated derivatives are highly enriched in mammalian retina. These fatty acids cannot be synthesized *de novo* in animal tissue and must be supplied in the diet or be synthesized from dietary linolenic acid (Bazan & Scott 1990). Thus, human and animal nutrition studies have focused on the essentiality of n-3 fatty acids in the normal physiology of photoreceptor cells. During development and/or during synaptogenesis and photoreceptor biogenesis, deficiency of these fatty acids has been shown to provoke profound biochemical changes in the fatty acid composition of the membrane phospholipids which results in functional changes (Neuringer et al., 1986; Weisinger et al., 1996a). These changes affect the balance between the supply of new molecules and the degradation of old molecules (Anderson et al., 1974). Formula with C22:6n-3 fed to preterm or term infants appear to have beneficial effect on visual function comparable to infants fed breast milk (Werkman & Carlson et al., 1996; Markrides et al., 1995). The structural and functional characteristics of retinas are sensitive to the light environment. Docosahexaenoic acid is reduced by constant or bright cyclic light (Wiegand et al., 1986; Penn and Anderson, 1987). This reduction may be related to rhodopsin bleaching and regeneration. In retinal diseases such as retinitis pigmentosa and diabetic retinopathy, the abnormal lipid metabolism causes severe membrane dysfunction which results in blindness (Converse et al., 1989; Simonelli et al., 1996).

Docosahexaenoic Acid Supply and Conservation

It is of interest how docosahexaenoic acid is supplied to the developing retina to meet both its requirement and to be conserved in such enriched amounts in the membrane. The intestine (Li et al., 1992) and liver (Scott & Bazan, 1989) appear to play an important role. Oral doses of [C^{14}]18:3n-3 and [C^{14}]22:6n-3 are esterified to triacylglycerols and phospholipids by the intestinal absorptive cells and transported in chylomicrons to the liver (Li et al., 1992). The liver synthesizes C22:6n-3 from C18:3n-3 and can deliver C22:6n-3 to the retina and brain via lipoproteins or albumin (Scott & Bazan, 1989). Phospholipid, triglyceride and cholesterol esterified with docosahexaenoic are carriers of docosahexaenoic acid to nervous tissues (Wang & Anderson, 1993b; Li et al., 1992; Martin et al., 1994). Retinal pigment epithelium is actively involved in the preferential uptake of docosahexaenoic acid from circulation and subsequently transports this fatty acid to the rod outer segment (Wang & Anderson, 1993a & 1993b). Wang et al. (1992) confirmed that retina and brain selectively takes up C22 fatty acid from the circulation since rats fed rapeseed oil (C22:1n-9, 43%) for 4 months from birth did not incorporate C22:1n-9 into the rod outer segment or brain but incorporate this fatty acid into other organs (Poulos et al., 1994). It is not understood how the nervous cells take up specific fatty acids. Bazan (Scott & Bazan, 1989; Bazan, 1990) suggests two basic mechanism in this regard: first, a signaling mechanism in the central nervous system which is sent to the liver to secrete docosahexaenoic acid containing lipoproteins; second, receptor mediated recognition mechanism that sequester docosahexaenoic acid from the blood. This pathway has not been confirmed.

During essential fatty acid deficiency, docosahexaenoic acid content in the retina and ROS, like the brain, are only slightly reduced (Wiegand et al., 1991; Anderson & Maude, 1971; Anderson et al., 1974). This indicates that there must be special mechanisms to conserve docosahexaenoic acid. Wiegand et al. (1991) suggested the conservation mechanisms in retina through recycling within the retina or between the pigment and the retina, or selective sequestration of docosahexaenoic acid from the blood. Since then many studies have confirmed the intimate involvement of retinal pigment epithelium by this mechanism (Stinson et al., 1991b; Chen et al., 1992; Gordon & Bazan, 1993). For example, docosahexaenoic acid increases in frog pigment epithelium following rod photoreceptor shedding and returns to the normal level within 8 hours following the shedding events (Chen et al., 1992). The phagosomal fatty acid labeling matches fatty acid composition of ROS tip (Gordon & Bazan, 1993) and C22:6n-3 containing lipids are released, removed and recycled back to the photoreceptors (Chen & Anderson 1993a; Gordon & Bazan, 1993). More recently, Chen and Anderson (1993b) found that docosahexaenoic acid is incorporated into triglyceride in the retinal pigment epithelial cell, suggesting that this neutral lipid participates in the selective enrichment of docosahexaenoic acid. Retinal pigment epithelium can be a source of C22:6n-3 for the retina since it is able to synthesize C22:6n-3 from precursors *in vitro* (Wang & Anderson, 1993a). Interphotoreceptor retinoid-binding protein (IRBP) located in the interphotoreceptor matrix may also be involved in the recycling of docosahexaenoic acid since this fatty acid shows the highest affinity for IRBP among all the fatty acids tested (Chen et al., 1993). There is a steep transient concentration of docosahexaenoic acid between photoreceptor and pigment epithelium cells (Chen et al., 1996).

Developmental Changes in Retinal Fatty Acids

It is important to know the developmental profile of fatty acids during the period of rapid nervous cell growth to determine the normal values during maturation. This can be an index for the intervention for disease or dietary treatment. Retinal fatty acid profiles change during maturation of both humans and animals (Bazan, 1988; Martinez et al., 1988; Martinez, 1988). From the prenatal period to adulthood, the retinal fatty acid composition shifts from saturated to unsaturated. More specifically, the level of docosahexaenoic acid increases and arachidonic acid decreases (Bazan 1988; Martinez et al., 1988). These changes are rapid during synaptogenesis and photoreceptor membrane biogenesis (Bazan, 1988). Similar changes occur in the brain and liver with maturation (Martinez, 1988; Jumpsen et al., 1997). Martinez (1988) suggests that the ratio of C22:6n-3/C20:4n-6 can be used as a marker of developmental changes in polyunsaturated fatty acid levels in retinal tissues . The phospholipid content of photoreceptors increase as a function of age in the mouse, whereas protein content remains relatively unchanged (Scott et al., 1988). Among the phospholipids, phosphatidylethanolamine and phosphatidylserine increased and phosphatidylcholine and phosphatidylinositol decreased. Jumpsen et al. (1997) found that docosahexaenoic acid increased with age in brain neuronal and glial cells isolated from cerebellum, frontal and hippocampus when rats are fed varying levels of n-6 and n-3 fatty acid. Therefore, the rapid accumulation of docosahexaenoic acid during maturation of visual cells may be related to the development of physiological function of the retina.

Dietary Modulation and Retinal Function

Dietary Modulation

During development, dietary n-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid, can greatly affect the fatty acid composition of membrane phospholipids in nervous tissues within a relatively short time (Philbrick et al., 1987; Foot et al., 1982; Hargreaves & Clandinin, 1987a, 1987b & 1987c). Effect of maternal diet on the modulation of n-3 fatty acid in retina of offspring has been studied in newly hatched chicks (Anderson et al., 1989), juvenile felines (Pawlosky et al., 1997) and piglets (Arbuckle & Innis, 1993). Docosahexaenoic acid appears to be the preferred fatty acid for raising the level of docosahexaenoic acid in retina and brain among different sources of n-3 fatty acids (Anderson et al., 1990). Feeding corn oil supplemented with docosahexaenoic acid is able to restore docosahexaenoic acid level in n-3 deficient chicks (Anderson & Connor, 1994) and felines (Pawlosky et al., 1997) probably through replacement of molecular species such as dipolyunsaturated (C22:6n-3 - C22:6n-3) (Lin et al., 1994). These observations imply that linolenic acid alone in the diet as the n-3 fatty acid source may not be adequate for normal development of the retina and brain, since C18:3n-3 is a less efficient precursor for docosahexaenoic acid when there is low Δ^6 desaturase activity (Anderson et al., 1990; Kohn et al., 1994). Su et al. (1996) found retinal unsaturated fatty acid levels paralled liver and plasma levels. Therefore, both liver and plasma may be a good estimator to predict the neural tissue membrane fatty acid composition. Feeding various amount of vegetable oil formula containing a high level of linoleic acid and a relatively low level of linolenic acid tend to maintain arachidonic acid but not docosahexaenoic acid in brain and retina (Hrboticky et al., 1991; Pawlosky et al., 1997). Breast fed infants have a greater proportion of DHA in their erythrocytes and brain cortex relative to those fed formula (Makrides et al., 1994). It has been suggested that optimal accretion of docosahexaenoic acid during the fetal and postnatal period may depend on placental transfer and an adequate dietary supply (Lin et al., 1991; Carlson et al., 1987; Clandinin et al., 1980a & 1980b). Feeding fish oil to rabbits (Lin et al., 1991) and guinea pigs (Weisinger et al., 1995 & 1996a) increased the docosahexaenoic acid level in retina, in spite of its normal high level of C22:5n-6. Caution, however, has to be given if the high level of C22:5n-6 in these animals is species-specific not just n-3 fatty acid deficient, as dietary modulation of n-3 fatty acid may not be appropriate in relation to their physiological functions.

Retinal Function

Although retina and ROS retain docosahexaenoic acid tenaciously during essential fatty acid deficiency (Wiegand et al., 1991; Connor et al., 1990 & 1991), severe unbalanced n-6/n-3 diet or depleted n-3 level in membrane can cause abnormal change in biochemical and physiological membrane function. Post malnourished infants, who had been fed very unbalanced n-6/n-3 since birth at 25 weeks of gestation, show significant decrease in retinal docosahexaenoic acid and increased C22:5n-6 (Martinez et al., 1988). The level of docosahexaenoic acid in n-3 fatty acid deficient chick brain and retina is restored not from feeding corn oil but a diet containing docosahexaenoic acid (Anderson & Conner, 1994). This result agrees well with studies of n-3 deficiency in the rhesus monkey (Neuringer et al., 1986; Neuringer & Connor, 1986). Functionally, monkeys show a delayed recovery of the dark adapted electroretinogram and impaired visual acuity at an early age (Neuringer et al., 1986), suggesting that n-6 fatty acids are not interchangeable with n-3 fatty acid in maintaining normal retinal function. After repletion with fatty acids from fish oil, docosahexaenoic acid level increased rapidly after feeding but no improvement in the electroretinogram occurred (Neuringer and Conner, 1989). Developing felines fed corn oil from gestation to 8 week old age are devoid of a dietary source of docosahexaenoic acid and display increased a and b wave implicit time compared to diets containing long chain fatty acids (Pawlosky, 1997). Guinea pigs fed safflower oil through three generations also exhibit significantly decreased levels of docosahexaenoic acid and reduced both peak to peak and a wave in the electroretinogram (Weisinger et al., 1996b). The same authors (1996a) found that retinal function in guinea pig is associated with docosahexaenoic acid with an optimal level of 19%. This suggests that although C22:6n-3 is essential in retinal visual function, supplying the appropriate amount has to be established for each species. Rats raised for several generations with a fat-free diet also show decreased amplitude of a wave, which reflects the electrical potential of the photoreceptor membrane, and altered ROS disc renewal (Anderson et al., 1974). These studies indicate that n-3 fatty acids exert a key role in normal visual function that is dependent on the n-6/n-3 balance in the diet. It also indicates that the most critical time for providing an adequate diet would be during pregnancy and early lactation. It would be of interest to see the effect of feeding n-3 fatty acids of high or low level within a practical range of n-6/n-3 ratio on retinal fatty acid composition and functions.

From the above studies, the importance of n-3 in nervous cells is obvious but the essentiality of docosahexaenoic acid is still questioned. Recently more research has focused on assessment of the infant's essential fatty acid status in relation to visual function in both term and preterm infants. These studies usually compare feeding breast milk versus infant formula with or without long chain fatty acids. Leaf et al. (1996) found correlation between % intake of breast milk (>50% versus <50%) and docosahexaenoic acid in both plasma and red blood cells. Uauy and coworkers (1990 & 1992) have reported that very low birth weight neonates fed a soybean oil based infant formula have poorer early electroretinogram response, and visual evoked potential (VEP) and forced choice preferential looking (FPL) compared to neonates fed human milk or marine oil containing formula. Preterm infants fed formula with marine oil (0.3% C20:5n-3 and 0.2% C22:6n-3) appear to have enhanced visual acuity measured by Teller Acuity Card procedure until 4 months of age (Carlson et al., 1993). With similar formula but using different tools of visual function measurement, Werkman & Carlson (1996) have demonstrated the beneficial effect of docosahexaenoic acid on discrete looks to both novel and familiar stimuli and short overlook duration the novelty preference (visual recognition memory). The preterm infant measured at 52 weeks of postconceptual age after feeding long chain fatty acid (0.3%, C22:6n-3) show the maturation pattern of visual evoked potential similar to breast fed infants (Faldella et al., 1996). All of the above studies exhibit the strong correlation between retinal function and red blood cell docosahexaenoic acid. Thus, docosahexaenoic acid in the red blood cell may be a good predictor of neural cell membrane (Carlson et al., 1993). However, if this is true, important questions arise since fatty acid composition of the visual cell is different from erythrocyte in terms of the high percentage of long chain fatty acids and the existence of very long chain fatty acids. Jongmans et al. (1996) found that infants born prematurely with the absence of other major neurological signs are at risk for abnormal visual function and perceptual-motor difficulties at earlier school life. From the above studies, it is clear that an appropriately balanced diet can positively affect visual function in infants born prematurely. Dietary intervention starting as early as possible would be beneficial.

Term infants fed breast milk for the first 4 months of life increase visual acuity measured by Teller acuity card more rapidly compared to formula fed infants (Jorgensen et al., 1996). This result is paralled by a decrease in C22:6n-3 in the red blood cells in formula fed infants. Carlson et al. (1996) have reported that term infants fed formula with 2% C18:3n-3 and 0.1% C22:6n-3 show better 2-month visual acuity than infants fed formula. The increase was transient. Markrides et al. (1995) also have found that visual evoked potential acuity of breast fed neonates and infants fed a formula supplemented with C22:6n-3 are better than those of placebo-formula fed infants at 16 and 30 weeks of age. Although these results show an effect of diet on the development of the visual function in a different group of infants, other studies have not given comparable results. For example, term infants fed formula (18:2n-6:18:3n-3=9.5:1) or (18:2n-6:18:3n-3=7.3:1) may have similar preferential looking acuity development during the first 3 months of life compared to infants fed breast milk (Innis et al., 1997). These data support the study of Auestad et al. (1997), who failed to demonstrate any relationship between dietary n-3 long chain fatty acid and visual acuity measured by sweep visual evoked potential and Teller acuity card. Based on these studies it is difficult to compare their results in term infants since dietary treatment and tools of assessment were different. Some studies were randomized and some were not. It is also possible that early exposure to light may have affected the developmental functions measured. It is clear that docosahexaenoic acid level in the plasma of infants fed formula supplemented with long chain fatty acids can be similar to breast fed levels. Many of these studies have based their physiological assessments on varied aspects of visual function. It would be also important to identify how biochemical function can be modulated with diet treatment using animal models.

Light

The structural and functional characteristics of retinas respond to the light environment. Animals can alter a combination of several retinal parameters in order to adapt to the illumination of their environment. For example, Schremser & Williams (1995a) noticed that soon after rats are switched to a new light intensity, the rod outer segment is always shortened regardless of raising the light intensity. There are two different theoretical mechanisms of light induced retinal damage. One is rhodopsin mediated since elevated rhodopsin levels correlate to increased damage susceptibility (Noell, 1980). The reason is that the action spectrum of light damage coincides with the absorbance spectrum of rhodopsin (Noell, 1980). The other is lipid mediated since lipid is photooxidized and produces peroxides causing the lesions of retina (Wiegand et al., 1983 & 1986; Organisciak et al., 1985). Light induces change in retinal rhodopsin levels and other parameters including rod outer segment length, photoreceptor cell number and outer nuclear layer thickness. These changes are inversely related with light intensity and duration (Penn & Williams, 1986; Schremer and Williams, 1995a; Penn et al., 1992). For example, rats raised in bright light compared to dim light contain a lower level of rhodopsin coupled with shortened rod outer segment length, reduced number of photoreceptors and thinner outer nuclear layer and reduced rhodopsin synthesis

(Schremser and Williams, 1995a & 1995b; Organisciak et al., 1996: Penn and Williams, 1986). When these animals are switched to dim light, those parameters are returned to the similar level of rats raised in dim light (Penn and Williams, 1986; Penn and Anderson, 1987). Therefore, Penn and Anderson (1987) introduced a concept that the changes in retinal parameters may be just the adaptation of the animal to its new light environment rather than light damage at least at the intensity of 800 lux. Schremser and Williams (1995a & 1995b) support this concept from their studies which demonstrate that light induced changes in retinal parameters are for the process of rod outer segment renewal adapting to a new intensity environment. The question of whether it is light damage or adaptation can be answered if the physiological light adaptation results in the reversibility of the parameters caused by light damage.

The importance of docosahexaenoic acid associated with photoreceptor membrane and its function has been mentioned in previous sections. The involvement of this fatty acid in light damage or adaptation has not been explored. Earlier studies have shown that docosahexaenoic acid in rat rod outer segment is reduced by constant illumination (1000-1300 lux) (Wiegand et al., 1983 & 1986) and bright cyclic illuminance (300 lux and 800 lux) (Penn and Anderson, 1987). Retinal phospholipid fatty acid molecular species, especially, C22:5n-6-C22:6n-3 and C22:6n-3-C22:6n-3 is reduced by a constant light (Wiegand et al., 1995). This exhibits a clear relationship between light exposure and docosahexaenoic acid. One possible hypothesis drawn from these results is that when rhodopsin is bleached, it may selectively take up docosahexaenoic acid to reduce the substrate for peroxidation, to prepare for visual pigment regeneration or to recycle for synthesizing new molecules containing C22:6n-3.

More recent studies have reported the effect of dietary fat on light response of the retina. Very recently, Organisciak et al. (1996) found that rats fed n-3 fatty acid (C18:3n-3) deficient diets exhibited protection against light induced retinal damage despite a higher content of rhodopsin. This supports the earlier study showing that rats fed a linseed diet are more susceptible to light damage than rats fed a chow diet (Organisciak et al., 1987). Reduced retinal docosahexaenoic acid by feeding safflower oil or coconut oil reduces intense light induced retinal degeneration (Bush et al., 1991) and is less susceptible to photoreceptor cell loss (Koutz et al., 1995). This protection may be related to a decrease in the oxidative potential of the rod outer segment. Light susceptibility is not enhanced in rats fed fish oil rich in C20:5n-3 and DHA (Reme et al., 1994). The authors suggest that a fish oil diet may change n-6/n-3 ratio of eicosanoids for less light induced disruption in basal ROS. It is puzzling to interpret the results from these studies in relation to the previous section (under 'Retinal Function') of this chapter. It is not known whether diet and light induced changes are a physiological adaptation and beneficial for retinal function since none of above studies measured electroretinograms. However, it is clear that both dietary fat and light history play a role in susceptibility of the retina to acute and chronic effects. Further studies are needed to elucidate the mechanism of docosahexaenoic acid involvement in light induced retinal parameters.

Retinal Disease

Retinitis Pigmentosa

Retinitis pigmentosa encompasses a group of progressive hereditary diseases which primarily affect photoreceptor and pigment epithelial function. The characteristic symptoms of retinitis pigmentosa are night blindness, low vision and restricted visual fields which ultimately lead to severe impairment of all retinal functions and blindness. This hereditary retinitis pigmentosa retinal degeneration has also been reported in animal species such as RCS rats, rd mouse, miniature poodle (progressive rod-cone degeneration, PRCD)(Anderson et al., 1991a), and Abyssinian cat (PRCD)(Anderson et al., 1991b). A biochemical marker in lipid metabolism for retinitis pigmentosa has been identified. Low docosahexaenoic acid levels in plasma has been reported in autosomal dominant retinitis pigmentosa (Newsome et al., 1988; Gong et al., 1992). Usher's syndrome which is progressive retinitis pigmentosa combined with a congenital hearing defect (Bazan et al., 1986b) and x-linked retinitis pigmentosa (McLachlan et al., 1990) also have low levels of docosahexaenoic acid in plasma. More recently this abnormality has also been identified in red blood cells in patients with retinitis pigmentosa (Schaefer et al., 1995; Simonelli et al., 1996; Hoffman et al., 1993). A remarkable increase of saturated fatty acids and a reduced polyunsaturated to saturated fatty acid ratio are noticed in retinitis pigmentosa affected erythrocytes and platelets (Stanzial et al., 1991). Similar changes in fatty acid levels are detected in animal models. Plasma lipids from progressive rod-cone degeneration affected miniature poodles (Anderson et al., 1991a) and the Abyssinian cat (Anderson et al., 1991b) show that docosahexaenoic acid is low but C22:5n-3 is increased, resulting in significant increase in the ratio of C22:5n-3/C22:6n-3. This suggests the possibility of a defect in the final stage of docosahexaenoic acid synthesis in animals affected with progressive rod-cone degeneration. This metabolic defect has also been identified in humans (Schaefer et al., 1995; Hoffman et al., 1995). However, docosahexaenoic acid synthesis from C22:5n-3 is not affected in retina and retinal pigment epithelium in miniature poodles (Alvarez et al., 1994). This suggests that docosahexaenoic acid synthesis may only be affected in red blood cells and/or that the transport of acyl groups from plasma and liver to tissue is affected in this disease state. However, biochemical studies of miniature poodles also show defective fatty acid esterification in ROS phospholipids in affected animals (Wetzel et al., 1989).

It would be interesting to see how this disease affects the retinal fatty acid composition. Higher levels of n-6 long chain fatty acid with decreased level of C22:5n-3 and C22:6n-3 has been reported in the retina of Swedish Briard dogs (Anderson et al., 1997). Scott et al. (1988) have shown that the developing rd mouse has a higher level of saturated and monounsaturated fatty acids and lower levels of polyunsaturated fatty acids in the retina (C20:4n-6 and C22:6n-3). The authors suggest that fatty acid metabolism is altered by expression of the rd gene and/or by the associated impairment of photoreceptor cell differentiation. Although there is no conclusion if abnormal lipid metabolism is the cause or effect in the symptom of retinitis pigmentosa, at least this abnormality of fatty acid composition may contribute to retinal integrity. It has been suggested that the low level of plasma docosahexaenoic acid is due not to a biochemical defect but to some extrinsic factor such as diet (McLachlan et al., 1990). A very recent study of progressive rod-cone degeneration in dogs after feeding docosahexaenoic acid enriched diet shows the level of docosahexaenoic acid is increased in plasma and liver but fails to increase the level in the rod outer segment. (Aguirre et al., 1997). The electroretinogram was not improved even after feeding C22:6n-3 for 21 weeks. The diet treatment in this experiment started when animals were young adults. Therefore it would be of interest to try dietary supplement of C22:6n-3 on retinal degeneration when animals are in an earlier developmental stage.

Diabetic Retinopathy

Diabetic retinopathy is one of the leading causes of blindness in people 20 to 74 years of age in the United States (Klein & Klein, 1984). This complication of diabetes mellitus is characterized by abnormal carbohydrate and lipid metabolism. It has been known that hypertension and abnormal retinal blood flow (Yoshida et al., 1983; Fallon et al., 1987) are associated with an increased risk of retinopathy. Chase and colleagues (1990) have reported that good maintenance of blood glucose levels and diastolic blood pressure below the 90th percentile helps reduce the risk of diabetic retinopathy.

Studies in the 1960's indicated the relationship between diabetes and fatty acid metabolism in retina. Retinal tissue of the alloxan treated diabetic dog showed marked depression in polyenoic fatty acid synthesis (Futterman et al., 1968b), resulting in an altered fatty acid composition of the retinal vasculature (Futterman et al., 1968a & 1969). Arachidonic acid and docosahexaenoic acid were decreased and the relative proportion of linoleic acid increased, indicating that retinal tissue is another target tissue for insulin action on synthesis of membrane components. Cultured retinal pigment epithelium from type 1 diabetics also show an altered insulin response (Miceli & Newsome 1991). It is perhaps relevant to note that Field et al. (1988) reported that the activity of the insulin receptor is affected by diet induced changes in plasma membrane phospholipid content and fatty acid composition of adipocytes.

Recently Gordon and coworkers (1991) indicated that retinal detachment alters the metabolic pathways involved in docosahexaenoic acid esterification into lipids. Incubation of detached diabetic retina with [^{3}H]22:6n-3 show alterations in the labelling pattern characterized by greater accumulation of free docosahexaenoic acid and label in triacylglycerol and decreasing label in phosphatidylcholine and phosphatidylinositol. These changes in [^{3}H]docosahexaenoic acid metabolism appear to be prior to the loss of photoreceptor cells and to a decrease in the endogenous docosahexaenoic acid containing phospholipid content. In contrast, the content of arachidonic acid containing phospholipid is unchanged as linoleic acid uptake is increased with retinal detachment. However, it is not clear whether the disrupted docosahexaenoic acid metabolism causes or results in diabetic detachment of the retina. It is possible that dietary n-3 treatment may affect membrane phospholipid fatty acyl esterification in the retina in relation to the diabetic state. A very recent study of feeding fish oil to streptozotocin treated rats for 6 months showed a two fold increase in n-3 fatty acid (C20:5n-3) in total plasma and erythrocyte. However diabetic associated retinal parameters such as pericyte numbers and occulded capillary segments became worse (Hammes et al., 1996). Since membrane composition of C22:6n-3 was not measured in this experiment, the correlation between docosahexaenoic acid and diabetic retinal parameters cannot be assumed, but could be hypothesized based on what we know about diet fat and its incorporation into tissues.

Chapter II. RESEARCH PLAN

Rationale

It is well known that a relationship exists between dietary fat composition and changes in membrane phospholipid content, composition and function (Clandinin et al., 1985; Hargreaves & Clandinin, 1987a & 1987b). Dietary n-3 fatty acids affect both the polyunsaturated fatty acid composition of membrane and a variety of functions.

Polyunsaturated fatty acids, especially docosahexaenoic acid, are highly enriched in retinal membrane phospholipids. The level of docosahexaenoic acid is increased with age, from infancy to adulthood, in both human and animal retina with decreasing arachidonic acid level. These changes are rapid during synaptogenesis and photoreceptor membrane biogenesis. The rapid accumulation of docosahexaenoic acid during maturation of visual cells must be related with the physiological function of the retina. Animals or human infants fed an unbalanced n-6/n-3 fatty acid diet or essential fatty acid deficient diet have shown altered retinal fatty acid composition which results in impaired visual cell renewal and abnormal visual function. Feeding fish oil as a source of n-3 fatty acids has reduced n-6 fatty acids in the retina while showing positive effects on visual function. These observations suggest that dietary C20 and C22 n-3 fatty acids may be of clinical benefit in retina not only for optimal maintenance of retinal membrane but also in retinal disease state. It would be of interest to identify the effect of feeding n-3 fatty acids at high or low levels within practical range of n-6/n-3 ratio on retinal fatty acid compositions and functions.

Docosapentaenoic and docosahexaenoic acid are generally considered to be the major end products of n-6 and n-3 fatty acid metabolism. Recently longer chain n-6 and n-3 very long chain fatty acids have been found in retinal membranes, specifically more in the rod outer segment. Current knowledge about the function and metabolism of these fatty acids is limited and remains to be elucidated. The effect of dietary fat intervention on the content and composition of these fatty acids has not been studied. It is reasonable to predict that increasing dietary n-3 fatty acid may alter the balance of n-6 and n-3 very long chain fatty acids in the retina since these fatty acids have to be elongated from their shorter chain precursors which can easily be altered by diet treatment. However, the identification of the precursor for the very long chain fatty acid is another question to be answered.

The structural and functional characteristics of the retina are sensitive to light exposure. Docosahexaenoic acid is reduced by constant or bright cyclic light. This reduction may be related to rhodopsin bleaching and regeneration as a response to the light environment. The involvement of dietary fat in the response to retinal fatty acid composition to light and rhodopsin function in the photoreceptor cell has not been elucidated. Thus study of the effect of membrane fatty acid composition in retinal function after diet and/or light treatment is merited.

Research Objectives

The main goal of the present study is to determine the effect of altering diet fat on membrane composition and rhodopsin function in the retina and specifically on the rod outer segment. Specific focus has been given to diet induced changes in very long chain fatty acids. The objectives of this research are:

1. to determine the effect of dietary n-3 fatty acid on membrane content of long and very long chain fatty acid in the rod outer segment.
2. to measure the effect of dietary n-3 fatty acid level and light exposure on long and very long chain fatty acid composition of visual cells.
3. to examine the influence of dietary fat on rhodopsin functions, such as rhodopsin content, rhodopsin disappearance kinetics, rhodopsin phosphorylation and rhodopsin regeneration.
4. to identify the effect of supplying varying levels of n-6 and n-3 fatty acids on membrane fatty acid composition, rhodopsin content and rhodopsin kinetics in developing photoreceptors.

5. to verify the influence of dietary fat on the synthesis of very long chain fatty acids of photoreceptors following light exposure.
6. to determine the preferred fatty acid precursor for synthesis of very long chain fatty acids.

Hypotheses

Dietary fat will induce change in the fatty acid profile of phospholipids and rhodopsin function of visual cells in response to light. It is specifically hypothesized that in photoreceptors of the retina:

1. The content of long and very long chain fatty acids of C24 to C36 n-6 and n-3 fatty acids can be altered by dietary intake of varying ratios of n-6/n-3 fatty acids in normal and diabetic animals.
    - 1-1. High n-3 fatty acid diet will increase the membrane content of n-3 long and very long chain fatty acids and decrease n-6 long and very long chain fatty acids.
    - 1-2. Dietary fat will alter the fatty acid profile of phospholipids of diabetic animals.
2. The profile of long and very long chain fatty acids of C24 to C36 n-6 and n-3 fatty acids will be altered by light exposure and can be affected by dietary intake of varying ratios of n-6/n-3 fatty acids.
3. The function of rhodopsin can be altered by dietary intake of varying ratios of n-6/n-3 fatty acids.
    - 3-1. High n-3 fatty acid diet will affect rhodopsin photolyzing kinetics and improve rhodopsin function.
    - 3-2. High n-3 fatty acid diet will increase rhodopsin content, rhodopsin regeneration and rhodopsin phosphorylation after light exposure.

4. Developmental profile of long and very long chain fatty acids of C24 to C36 n-6 and n-3 fatty acids will increase with age in growing animals and will be affected by dietary fat intake.
    - 4-1. Supplementing arachidonic acid in the diet will increase the level of C20:4n-6 and n-6 very long chain fatty acid of phospholipids in developing rod outer segment.
    - 4-2. Supplementing docosahexaenoic acid in the diet will increase the level of C22:6n-3 and n-3 very long chain fatty acids of phospholipids in developing rod outer segment.
    - 4-3. Feeding a mixture of arachidonic acid and docosahexaenoic acid will increase both n-6 and n-3 long and very long chain fatty acids of phospholipids in developing rod outer segment.
    - 4-4. Increased linolenic acid in the diet will increase n-3 long and very long chain fatty acids while decreasing n-6 long and very long chain fatty acids.
    - 4-5. Feeding diets containing long chain fatty acids will increase the level of rhodopsin in the developing retina.
5. The synthesis of long and very long chain fatty acids of C24 to C36 n-6 and n-3 fatty acid will be altered by dietary intake of varying ratios of n-6/n-3 fatty acids.
    - 5-1. C20:5n-3 but not C22:6n-3 is the substrate for synthesis of very long chain fatty acids in phosphatidylcholine.

Chapter Format

The hypotheses posed are tested in a sequence of experiments. These experiments are organized in chapters as follows:

Chapter III describes the methods used for retina and rod outer segment preparations.

Chapter IV tests hypothesis 1.

The effect of a high n-3 fatty acid diet (5.8% w/w, n-6/n-3=2.7) versus a low n-3 fatty acid diet (1.2% w/w, n-6/n-3=13.8) in the diet on the fatty acyl composition of the major membrane phospholipids of the rod outer segment is examined. The high fat diet (20% w/w) providing either high (C22:6n-3 1% w/w) or low (C18:3n-3 1% w/w) content of n-3 fatty acid in a diet with a low polyunsaturated to saturated fatty acid ratio (P/S=0.27) are fed from weanling to 6 weeks old control and streptozotocin-induced diabetic rats (hypothesis 1-1 & 1-2).

Chapter V tests hypothesis 2 and 3.

The effect of dietary fat and light on long and very long chain fatty acid composition (hypothesis 2), rhodopsin content and rhodopsin phosphorylation of rod outer segment are measured (hypothesis 3-2) using rapidly growing weanling rats. Dietary fat supplying high n-3 fatty acid (4.8% w/w, n-6/n-3=2.5) containing C22:6n-3 (3.4% w/w) or n-3 fatty acid (1.2% w/w, n-6/n-3=13.6) containing no C22:6n-3 are fed to weanling rats under a 12/12 hour cyclic light. After 6 weeks of feeding, half of the animals in each diet group are exposed to either 48 hours of continuous light (1000 lux) or kept in complete darkness.

Chapter VI also tests hypothesis 3.

Effect of dietary fat and light on rhodopsin content, rhodopsin disappearance kinetics and rhodopsin regeneration *in vivo* and *in vitro* are determined in the entire retina (Hypothesis 4) using the same dietary fat and light regime as described in Chapter V.

Chapter VII tests hypothesis 4.

Effect of supplying small amounts of C20:4n-6 and C22:6n-3 on long and very long chain fatty acid composition of photoreceptor cells (hypothesis 4-1 to 4-4) and rhodopsin content and rhodopsin disappearance kinetics (hypothesis 4-5) are examined in 2, 3 and 6 week old rats. Diet fats are formulated to reflect the fat composition of a conventional infant formula providing a C18:2n-6 to C18:3n-3 ratio of 7:1. Preformed C20:4n-6 and /or C22:6n-3 are added in a physiological level to this fat blend. Another diet is formulated to increase the level of C18:3n-3 to provide a C18:2n-6 to C18:3n-3 ratio of 4:1. These formulated diets with or without C20:4n-6 and/or C22:6n-3 are used to determine whether preformed C20:4n-6 and C22:6n-3 provide an optimum level of long and very long chain fatty acids required by the growing photoreceptors compared to diets containing the precursors of C20:4n-6 and C22:6n-3.

Chapter VIII tests hypothesis 5.

The effect of dietary fat and light on the synthesis of n-3 long and very long chain fatty acid composition of photoreceptors *in vivo* is determined using ^{3}H-20:5n-3 and ^{3}H-22:6n-3 as substrates (Hypothesis 7). A high fat (20% w/w) diet with 1% (w/w) of either C18:3n-3 or C22:6n-3 in a low P/S (0.22) were fed for 6 weeks. The light condition is the same as described in Chapter V. The metabolic fate of ^{3}H-20:5n-3 is compared with ^{3}H-22:6n-3 (Hypothesis 5-1) by measuring the incorporation of these substrates into long and very long chain fatty acid of individual phospholipids.

Chapter IX provides a general summary and discussion.

Aspects of this data have been presented in paper format:

Chapter IV.

Suh M, Wierzbicki AA & Clandinin MT (1994) Dietary fat alters membrane composition in rod outer segments in normal and diabetic rats: Impact of content of very long chain (C≥24) polyenoic acids. Biochim Biophys Acta 1214, 54-62.

(Selected data from this paper have also been presented in)
Clandinin MT, Suh M & Hargreaves K (1992) Impact of dietary fatty acid balance on membrane structure and function of neural tissues. Neurobiology of Essential fatty acids. Bazan NG (Ed) Plenum press, New York. pp 197-210.

Suh M, Wierzbicki AA & Clandinin MT (1992) Dietary modulation of membrane content of very long chain fatty acids (VLCFA C>24) in photoreceptors of normal and diabetic rats. In Advances in Polyunsaturated fatty acid Research (Yasugi, T., Nakamura, H., and Soma, M. eds) Proceeding of the 5th Scientific Meeting of the Society for Research on Polyunsaturated fatty acids, Tokyo, Japan, Nov.1992. Excerpta Medica, International Congress Series 1025, pp197-200. The Netherlands: Elsevier Science Publishers.

Clandinin MT, Jumpsen J and Suh M (1994) Relationship between fatty acid, membrane composition and biological functions J Pediatr Suppl 125:S25-32.

Chapter V.
Suh M, Wierzbicki AA and Clandinin MT (1998) Dietary fatty acid modulate the content of long and very long chain fatty acids (C24-C36), rhodopsin content and rhodopsin phosphorylation in rat photoreceptor cells after light exposure. J Biol Chem (in preparation).

Chapter VI.
Suh M and Clandinin MT (1998) Dietary n-3 fatty acids alter rhodopsin content and rhodopsin regeneration in rat retina following light exposure. Biochim Biophys Acta (in preparation).

Chapter VII.
Suh M, Wierzbicki AA and Clandinin MT (1998) Dietary polyenoic fatty acids modulate the profile of long and very long chain fatty acids (C24-C36), rhodopsin content and kinetics in developing photoreceptor cells. J Pediatr (in prerparation)

(Parts of data from this chapter have also been presented in)
Suh M, Wierzbicki AA, Lien E & Clandinin MT (1996) Relationship between dietary supply of long-chain fatty acids and membrane composition of long and very long chain essential fatty acids in developing rat photoreceptors. Lipids 31, 61-64.

Chapter VIII.
Suh M, Wierzbicki AA and Clandinin MT (1998) C20:5n-3 is a preferred substrate for synthesis of n-3 very long chain fatty acids (C24-C36) in retina. Biochem J (in preparation).

Chapter III. METHODS

This chapter describes the methods for the retina and rod outer segment preparation used in subsequent chapters.

Sample Preparations and its Purity

Isolation of Retina

Retinas were isolated by the method of Fumiyuki and La Vail (1989). The cornea was pierced and dissected with the tip of a razor blade. Using forceps, slight pressure was applied to the eye cup enabling the lens and vitreous body to be easily extracted. While the lens was extruded, the retina which is connected to the lens by zonular fibers came out with the lens (Fumiyuki et al., 1989). The retina was then detached from the lens and was washed two or three times with 2 or 3 drops of cold saline solution (4°C). After examining histologically, the retinas were found to be morphologically intact with photoreceptors (see Appendix A-1).

Isolation of Rod Outer Segments (ROS)

ROS were isolated by discontinuous sucrose gradient centrifugation developed by Stinson & Anderson (1991a). All isolations were performed at 4°C. Ten isolated retinas were gently homogenized by hand with a glass homogenizer in 6 ml of 1.75 g/ml sucrose buffered with 10 mM Tris (pH 7.4) containing 70 mM NaCl, 2 mM $MgCl_2$ and 0.1 mM EGTA. The homogenate was transferred to the bottom of an ultra-clear centrifuge tube (Beckman 38.5 ml). Six ml of 1.140 g/ml sucrose was overlaid followed by 5 ml of 1.115 g/ml sucrose. These latter two sucrose solutions were buffered with 10 mM Tris-acetate (pH 7.4) and contained 0.2 mM $MgCl_2$ and 0.1 mM EGTA. Tubes were centrifuged at 82,000 X g for one hour at 4°C in a Beckman Model L8-70M centrifuge equipped with a type 60 Ti fixed angle rotor. The ROS layer at the 1.115-1.140 g/ml sucrose band interface was removed with a pasteur pipette and washed twice with 10 ml of 50 mM Tris-acetate (pH 7.4) containing 5 mM $MgCl_2$ and 0.1 mM EDTA at 27,000 X g for 20 min. The resulting pellet was placed directly into 10 ml of chloroform: methanol (2:1 v/v). The retinal cell body (CB) which remained in the tube after the ROS had been removed was mixed with 5 ml of Tris-acetate Buffer (pH 7.4) and centrifuged at 10,000 X g for one hour. This CB pellet contained all other membrane fractions from the whole retina. Aliquots of both ROS and CB fractions were taken and evaluated by polyacrylamide gel electrophoresis and microscopy.

Membrane Purity

Polyacrylamide Gel Electrophoresis (PAGE)

Twelve percent acrylamide containing sodium dodecyl sulfate was used for rhodopsin identification, using a Studier slab gel electrophoresis unit (SE 400, Hoefer Scientific Instruments, San Francisco, California) by the method of Laemmli (1970). Prepared aliquots of ROS and CB containing 262 ug and 374 ug protein respectively were solubilized in 1.6% (w/v) $NaDodSO_4$ with 0.05 M Tris-HCl (pH 6.8), 8% glycerol, 4% 2-β-mercaptoethanol and 0.001% bromophenol blue as a tracking dye and heated at 95°C for 4 minutes. The separating gel contained 12% acrylamide buffered with 0.375 M Tris-HCl (pH 8.8) and 0.1% (w/v) $NaDodSO_4$. The acrylamide: N,N'-methylenebis ratio was 29.2: 0.8. The stacking gel contained 7% acrylamide in 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) $NaDodSO_4$. For both gels, polymerization was initiated by the addition of 0.05% (w/v) ammonium persulfate and 0.01% (v/v) TEMED. Eighty microlitres of each solubilizing sample buffer, containing 42 ug (ROS) and 60 ug (CB) protein was loaded in each lane and was compared to appropriate molecular weight standards (MW-sds-200 Kit, Sigma Chemical Co., St. Louis, USA). The gel was 1.5 mm thick and electrophoresis was carried out at a constant current of 30 mA with a 100 watt voltage for 6 hours with electrode buffer (pH 8.3) containing 0.3% Tris, 1.4% glycine and 0.1% $NaDodSO_4$. Gels were stained for 12 hours in 0.2% (w/v) Comassie blue R-250, 40% (v/v) methanol, 10% (v/v) acetic acid and destained in 40% (v/v) methanol, 10% acetic acid for 12 hours or until the background was clear. Protein concentration was determined by the method of Lowry (1951) with bovine serum albumin as a standard.

Rod outer segments were isolated from entire retina and checked for its purity by polyacrylamide gel electrophoresis as shown in Figure A-3 (see appendix). As observed in the gel, opsin with a molecular weight of 35,000 Daltons (Hsieh & Anderson, 1975) was present in rod outer segment membranes. The retinal CB fraction shows heterogenous protein distribution with a small component having a molecular weight similar to that of opsin. In comparison with the total protein of retinal CB, the small amount of opsin indicates that rod outer segments can only be a minor contaminant of the retinal debris.

Microscopy

Microscopy was performed as a measure of membrane purity. Isolated retinas, ROS and CB were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) containing 1% sucrose for 24 hours. The pellet was dehydrated with increasing ethanol concentrations from 30% up to 100% and embedded in historesin. Samples were sectioned and examined with X 100 oil immersion optics (X625).

Microscopy of the entire retina, rod outer segment and retinal cell body are shown in Figure A-2 (see Appendix). The retina isolated by the method of Fumiyuki and Le Vail (1980) was composed of its distinct cell types and morphologically intact with photoreceptor cells without contamination of retinal pigment epithelia. Intact cells of rod outer segments were not seen in Figure A-2, since they were gently homogenized with a hand homogenizer before further separations by discontinuous sucrose gradient centrifugation. However, rod outer segment in Figure A-2 (A) is different in structure as compared to retinal cell body illustrated in Figure A-2 (B). It was concluded that the rod outer segment fraction contains little subcellular contamination.

Chapter IV.

Dietary Fat Alters Membrane Composition in Rod Outer Segments in Normal and Diabetic Rats: Impact on Content of Very Long Chain (C≥24) Polyenoic Fatty Acids

INTRODUCTION

Recent understanding of membrane lipid composition has been extended with discovery of very long chain fatty acids with chain lengths greater than 22 carbons. The content of these very long chain fatty acids is known only for a few specialized tissue lipids such as rod photoreceptors of the retina (Aveldano, 1987 & 1988) sperm, testes (Grogan et al., 1984; Poulos et al., 1986b) and brain, particularly in patients with peroxisomal diseases (Poulos et al., 1986a & 1988). Their function or physiological role is unknown. In photoreceptor membranes, the very long chain fatty acids may be associated with rhodopsin movement (Aveldano, 1988) and may play an important role in vision. Current knowledge concerning synthesis, membrane levels and specific functions of polyunsaturated very long chain fatty acids is extremely limited. The effect of dietary intervention on membrane content of these fatty acids has not been studied nor is the effect of diet fat on membrane composition in the retina known.

Considerable attention has focused on dietary polyunsaturated fatty acid composition because of the dynamic capability to alter membrane fatty acid composition (Bourre et al., 1988; Clandinin et al., 1985; Innis & Clandinin, 1981) which ultimately influences a variety of membrane functions (Clandinin et al., 1991). Although rod outer segments (ROS) of the retina contain a high level of C22:6n-3, few studies in human or animal nutrition have focused on the essentiality of n-3 fatty acids in the normal physiology or membrane composition of photoreceptor cells. During development, synaptogenesis and photoreceptor biogenesis, deficiency of C22:6n-3 provokes profound biochemical changes in composition of membrane phospholipids resulting in functional changes (Neuringer & Conner, 1986; Reddy & Bazan, 1985) that affect the balance between the supply of new molecules and degradation of old molecules (Anderson et al., 1974). Unbalanced n-6/n-3 diets can also cause abnormal biochemical and physiological membrane functions such as delayed recovery of the dark adapted electroretinogram and impaired visual acuity at an early age (Martinez, 1988; Neuringer & Conner, 1986). Feeding fish oil as a source of n-3 fatty acids shows positive effects on visual function (Carlson, 1988; Uauy et al., 1990 & 1992), but might be expected to reduce n-6 fatty acid content in the retina. Collectively, these studies indicate that n-3 fatty acids exert a key role in normal visual function that may be dependent on the n-6/n-3 balance in the diet.

The diabetic state influences the fatty acid profile of several tissue membranes in both human and streptozotocin-induced diabetic rats (Field et al., 1990), but the effect of the early diabetic state on ROS components has not been determined.

It is reasonable to predict that increasing dietary n-3 fatty acid may change the balance between the n-6 and n-3 very long chain fatty acids in the ROS if these fatty acids are elongated from shorter chain precursors that can be altered by diet treatment. This study was therefore carried out to determine the effect of altering diet fat composition on the long chain fatty acid and VLCFA content of individual phospholipids of the ROS in normal and diabetic animals.

MATERIALS AND METHODS

Animals and Diets

One hundred and twenty weaning male Sprague-Dawley rats (53.6 ± 1.1 g) were randomly assigned to two diet treatments and were fed semipurified diets (Field et al., 1988; see Appendix Table A-1) containing 20% (w/w) fat providing a low ratio of polyunsaturated to saturated fatty acids (P/S=0.27). Diets differed in level of n-3 fatty acids (high, 5.8% w/w or low, 1.2% w/w). Fish oil was used as the source of n-3 fatty acids in the high n-3 fatty acid diet while linseed oil was used in the low n-3 fatty acid diet. Fatty acid composition of diets fed is illustrated (Table IV-1) and generally reflects a range of fatty acid balance normally attained in the human diet.

Table IV-1. Fatty acid composition of diets.

Diet	High n-3	Low n-3
Fat source (g/kg)		
Beef tallow	145.0	160.0
Safflower oil	34.0	37.0
Linseed oil	-	3.0
Fish oil	21.0	-
Fatty acid (% w/w)		
C14:0	4.8	4.6
C16:0	25.0	27.7
C18:0	39.5	39.1
C18:1n-9+n-7	4.5	7.8
C18:2n-6	15.4	16.6
C18:3n-3	0.4	1.2
C18:4n-3	0.8	-
C20:0	0.4	0.4
C20:5n-3	3.2	-
C22:5n-3	0.3	-
C22:6n-3	1.2	-
n-6/n-3 ratio	2.7	13.8

Fatty acids with chain length greater than C24 were not detected in the diet.

After three weeks of feeding, diabetes was induced in half of the animals in each diet treatment by intravenous tail injection of streptozotocin (50 mg/kg body weight; Sigma Chemical Co., Canada) in an acetate buffer, pH 4.5. Animals were fed diets for an additional 3 weeks. Animals were housed individually in a controlled temperature of 21°C with a 12 hour light/dark cycle. Diets were prepared weekly and changed every other day. Food intake was measured daily and body weight recorded weekly. Animals were considered diabetic if non-fasting blood glucose levels were greater than 320 mg/dl. Animals were killed by decapitation after 1 hour of dark cycle. Eyeballs were immediately excised and blood was collected after killing for plasma glucose and insulin determination. Serum glucose was determined using a glucose analyzer (YSI Model 27 Industrial Analyzer, YSI Instrument Co., Yellow Springs, Ohio). Serum insulin levels were determined by radioimmunoassay (Insulin RIA 100 kit, Pharmacia Co., Uppsala, Sweden) against a rat insulin standard.

Isolation of Retina and Rod Outer Segments (ROS)

All isolations were performed at 4°C. Retinas were isolated (Fumiyuki et al., 1989). Ten retinas were pooled for separation of the ROS. ROS were isolated by discontinuous sucrose gradient centrifugation (Stinson et al., 1991a). Aliquots of both ROS and retinal debris were taken and evaluated by microscopy and polyacrylamide gel electrophoresis (Laemmli, 1970). In the gel, opsin with a molecular weight of 35,000 daltons was present in ROS membranes. The purified ROS fraction contained only minor subcellular contamination by retinal debris.

Lipid Analysis

Total lipids were extracted from the ROS (Folch et al., 1957). Phospholipids were separated from neutral lipids using silica G-plates (20 x 20 cm) and developed in a solvent system composed of petroleum ether:diethyl ether:acetic acid (80:20:1, by vol) and then eluted three times with 10 ml chloroform:methanol (1:1, v/v) containing 1 ppm ethoxyquin. Phospholipids were dried under nitrogen and redissolved in 0.2 ml hexane. Phospholipids were separated by spotting on hexane-prewashed silica gel H-plates (20 x 20 cm) developed in chloroform:methanol:2-propanol:0.25% (w/v) KCl:triethylamine (30:9:25:6:18, by vol)(Touchstone et al., 1980) for 1 1/2 hours followed by chloroform:methanol:1-propanol:0.25% (w/v) KCl:triethylamine (30:9:25:6:18, by vol) for 1 1/2 hours. With these two systems phosphatidylcholine was separated from sphingomyelin. TLC plates were dried at room temperature for 5 min and visualized with 0.01% (w/v) anilino naphthalene sulfonic acid in water. Each phospholipid was recovered and fatty acid methyl esters were prepared using BF_3/methanol reagent (Morrison & Smith, 1964).

Analysis of Fatty Acid Methyl Esters

Separation of long and very long chain fatty acid methyl esters was performed by gas-liquid chromatography using BP20 and BP1 fused silica capillary columns, respectively.

***Separation of medium and long chain fatty acids*:** A Varian Vista 6000 GLC equipped with a data system, DS 654, was used for separation of medium and long chain fatty acid methyl esters, utilizing a fused silica BP20 capillary column (25 m x 0.25 mm I.D.; Varian, Georgetown, Ontario, Canada). Helium was used as a carrier gas at a flow rate of 1.8 ml/min with inlet splitter set at 100:1. The initial oven temperature was 90°C, increased to 172°C at 20°C/min and held for 14.5 min followed by an increase in temperature to 220°C at 2.5°C/min and then held for 13 min. The injector and detector temperature was 250°C. With these conditions, all saturated, *cis*- mono-, di-, and polyunsaturated fatty acids from C12 to C24 were separated. Identification of peaks was based upon their relative retention times and equivalent chain lengths compared to known standards.

Separation of very long chain fatty acids: For separation of very long chain fatty acids, a Hewlett Packard 5890 GLC equipped with Vista DS 654 data system was used. Chromatography utilized a nonpolar BP1 column (12 m x 0.22 mm I.D.). The carrier gas was helium, at a flow rate of 2.2 ml/min and a split ratio of 20:1. The initial oven temperature was started at 110°C, increased to 320°C at a rate of 3°C/min and then held for 5 min. The injector temperature was 250°C. With these conditions, very long chain fatty acids up to 34 carbons in chain length were separated. Identification of the longer chain length fatty acids was based in part on retention times. Identification of the degree of unsaturation of the fatty acid was based on GC-MS analysis and prior separation of fatty acid methyl esters on the basis of number of double bonds by argentation TLC.

GC-MS Analysis

Fatty acid methyl esters were injected into the BP1 column and analyzed by an electron impact mass selective detector. The injector temperature was 250°C and the GC-MS transfer line temperature was 300°C. The identification of very long chain fatty acids was performed using a Hewlett Packard 5970 mass selective detector with a Hewlett

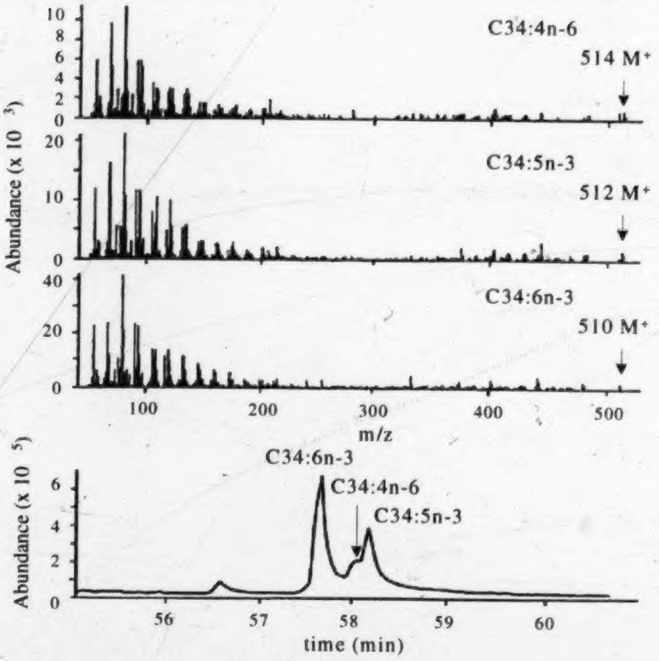

Figure IV-1. Electron impact mass spectra and total ion chromatogram of C34:4n-6, C34:5n-3 and C34:6n-3 methyl esters from phosphatidylcholine in rat rod outer segment.

Packard comp 216 data system. The mass spectra of very long chain tetra-, penta- and hexaenoic methyl esters from phosphatidylcholine contained patterns similar to that shown in Figure IV-1.

Double Bond Separation by Argentation TLC

Argentation TLC was used to resolve fatty acid methyl esters on the basis of degree of unsaturation. Samples were applied on TLC plates impregnated with $AgNO_3$. Analtech silica Gel H-plates were developed in a 10% (w/v) solution of $AgNO_3$ in water in a TLC tank for 60 min. TLC plates were dried for 3 min in the dark and activated in a 110°C oven for 1 hour. Each sample was spotted on the plate in a narrow band. Appropriate standards on plates were spotted and the plates were developed twice in a solvent system of hexane:diethylether:acetic acid:toluene:acetone (50:4:2:40:4, by vol) for 1 hour and then developed a second time in the same solvent for 30 min. Plates were dried at room temperature for 3 min and visualized with 0.1% (w/v) 2'7' dichlorofluorescein in 95% (w/v) ethanol. A typical chromatogram of fatty acid methyl esters separated by argentation TLC of standard and retinal phospholipids are in Figure A-4 (see Appendix). Each band was eluted sequentially with the following solvents: 2 ml hexane and 0.5 ml 0.01% NH_3OH, 2 ml hexane:diethyl ether:acetic acid (94:4:2, by vol) and 2 ml hexane. Each extract eluted was pooled and dried under nitrogen. Methyl esters of each band were injected into both GLC columns as previously described and fatty acid identity was confirmed by molecular ion (M^+) identification in the mass spectra of the GC-MS.

This GC-MS technique provided definitive identification of the series of fatty acids for polyenoic fatty acids containing diagnostic ions with m/z 79 as the base peak. Tetraenoic acids had a higher ion intensity for m/z 150, 164 and M-71, confirming that these belong to the n-6 series of fatty acids. Pentaenoic and hexaenoic n-3 series fatty acids were enriched in the diagnostic ions m/z 108, 131, M-29 and M-69. In addition to m/z 108 for n-3 and m/z 150 for n-6 series (Fellenberg et al., 1987), m/z 164 and M-71 for n-6 and m/z 131, M-69 and M-29 for n-3 series were also reliable diagnostic ions. The peaks indicated by arrows in Figure IV-2 also serve to verify the confirmation of the fatty acid series. For example feeding a high n-3 fatty acid diet increased n-3 pentaenoic and hexaenoic acids while decreasing n-6 tetraenoic acids compared to feeding a low n-3 fatty acid diet.

Statistical Analysis

The effect of diet and disease state on fatty acid composition of phospholipid in ROS were assessed by two-way analysis of variance procedures for unbalanced data. Significant effects of treatment were defined utilizing Duncan's multiple range test (Steel & Torrie, 1990). All data is expressed as mean ± standard error of the mean (S.E.M.).

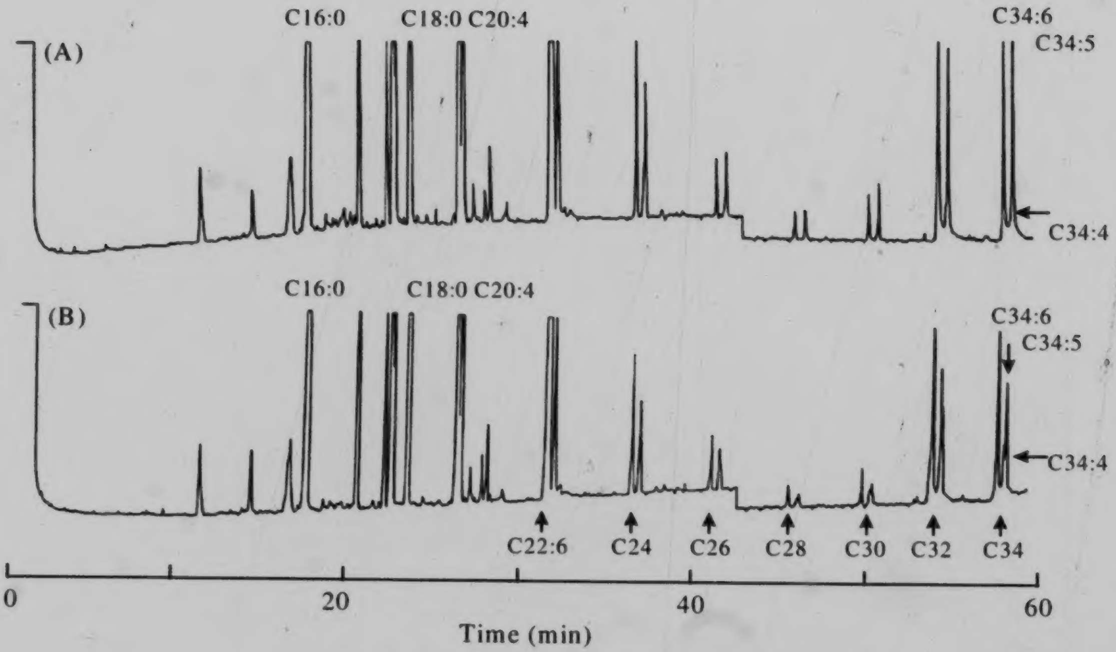

Figure IV-2. Typical chromatogram of very long chain fatty acid methyl esters separated by gas-liquid chromatography using a BP1 column. Chromatograms are from phosphatidylcholine of ROS fed either high n-3 (A) or low n-3 (B) fatty acid diet. The fatty acid peaks indicated by the arrows in C34 are an example of the reflection of diet treatment.

RESULTS

Weight gain during the final 3 weeks of feeding and serum insulin level was significantly lower in diabetic animals (2.8 g/d, 26.8 µU/ml) compared to diet-matched control animals (6.9 g/d, 54.0 µU/ml). Postprandial serum glucose level was significantly higher in diabetic animals (552.6 mg/dl) compared to control animals (124.6 mg/dl). No diet treatment effect was observed on weight gain and serum glucose or insulin levels.

Effect of Diet on Long and Very Long Chain Fatty Acid (C≥24) Composition of Rod Outer Segment Phospholipids in Nondiabetic Rats

Diet fat altered the fatty acid composition of major ROS phospholipids. Diet did not alter total saturated and monounsaturated fatty acid content in all phospholipid classes. Therefore, the fatty acid composition of each phospholipid is illustrated for only n-6 and n-3 fatty acids (Table IV-2 to IV-5).

Phosphatidylcholine

Only phosphatidylcholine contained very long chain polyenoic fatty acids up to carbon chain length 34[illegible]th 4, 5 and 6 double bonds. The level of these fatty acids was approximately 6.7% (w[illegible]of total fatty acids (Table IV-2). The major saturated fatty acid in this phospholipid was C16:0 (43% w/w) in comparison with other phospholipids having C18:0 (data not shown). Feeding a high n-3 fatty acid diet increased docosapentaenoic acid (C22:5n-3) and docosahexaenoic acid (C22:6n-3) (Table IV-2). Levels of all n-6 tetraenoic acids from 24 to 34 carbon atoms were significantly altered by diet treatment (Table IV-2 and Figure IV-2). Increased diet n-3 content depleted these n-6 fatty acids with increase observed in C24:5n-3. Levels of C28:5n-3, C30:5n-3, C32:5n-3 and C34:5n-3 were slightly higher in animals fed a high n-3 fatty acid diet compared to animals fed the low n-3 fatty acid diet, although the differences were not statistically significant. The feeding regimen was not associated with alterations in n-3 very long chain hexaenoic fatty acids from C24:6n-3 to C34:6n-3. However, the balance of total n-

6 to n-3 fatty acids in ROS for animals fed a high n-3 fatty acid was significantly lower compared to animals fed the low n-3 fatty acid diet.

Table IV-2. Effect of diet on long and very long chain fatty acid (C≥24) composition of phosphatidylcholine in rod outer segment.

Diet		High n-3 (n=5)	Low n-3 (n=3)	Significance level (p<)
Fatty acids	(% w/w)			
n-6[a]	C18:2	0.85 ± 0.09	0.83 ± 0.27	
	C20:4	4.03 ± 0.22	4.49 ± 1.08	
	C22:4	0.45 ± 0.13	0.44 ± 0.05	
	C22:5	0.16 ± 0.05	0.85 ± 0.11	0.001
n-3[b]	C20:5	0.43 ± 0.24	0.24 ± 0.19	
	C22:5	0.51 ± 0.02	0.21 ± 0.03	0.001
	C22:6	20.26 ± 0.75	12.27 ± 1.68	0.05
n-6 VLFCA[c]	C24:4	n.d.[f]	0.47 ± 0.08	0.001
	C26:4	0.01 ± 0.01	0.27 ± 0.03	0.001
	C28:4	n.d.	0.07 ± 0.01	0.001
	C30:4	n.d.	0.10 ± 0.01	0.001
	C32:4	0.04 ± 0.03	0.68 ± 0.08	0.001
	C34:4	0.02 ± 0.02	0.28 ± 0.04	0.001
n-3 VLCFA[d]	C24:5	0.42 ± 0.05	0.15 ± 0.09	0.01
	C26:5	0.23 ± 0.02	0.16 ± 0.01	0.05
	C28:5	0.12 ± 0.02	0.08 ± 0.06	
	C30:5	0.21 ± 0.05	0.13 ± 0.01	
	C32:5	0.80 ± 0.21	0.48 ± 0.05	
	C34:5	0.81 ± 0.29	0.66 ± 0.07	
	C24:6	0.62 ± 0.06	0.79 ± 0.13	
	C26:6	0.18 ± 0.04	0.27 ± 0.04	
	C28:6	0.09 ± 0.03	0.14 ± 0.01	
	C30:6	0.18 ± 0.07	0.20 ± 0.02	
	C32:6	1.44 ± 0.41	2.11 ± 0.31	
	C34:6	0.86 ± 0.22	1.24 ± 0.18	
Unsat. Index[e]		195.9 ± 11.1	157.6 ± 15.7	

Values given are means ± S.E.M. For each n 10 retinas were pooled. [a]n-6, n-6 long chain fatty acids; [b]n-3, n-3 long chain fatty acids; [c] n-6 VLCFA, n-6 very long chain fatty acids; [c] n-3 VLCFA, n-3 very long chain fatty acids; [e]Unsat. Index, Unsaturated index (number of double bond x % of each fatty acids); [f] n.d., not detectable. The content of very long chain fatty acid is approx. 7.2% (w/w) of total fatty acids.

Table IV-3. Effect of diet on long and very long chain fatty acid composition of phosphatidylethanolamine in rod outer segment.

Diet		High n-3 (n=5)	Low n-3 (n=5)	Significance level (p<)
Fatty acids	(% w/w)			
n-6[a]	C18:2	0.71 ± 0.09	1.23 ± 0.19	
	C20:3	0.26 ± 0.02	0.16 ± 0.08	
	C20:4	8.33 ± 0.24	12.46 ± 1.52	0.01
	C22:4	1.33 ± 0.11	2.31 ± 0.20	0.001
	C22:5	0.19 ± 0.01	2.05 ± 0.37	0.001
n-3[b]	C20:5	0.32 ± 0.05	0.24 ± 0.21	
	C22:5	0.84 ± 0.21	0.83 ± 0.17	0.05
	C22:6	39.59 ± 2.60	28.46 ± 2.45	0.01
VLCFA[c]	C24:4n-6	0.05 ± 0.01	1.01 ± 0.51	0.05
	C24:5n-3	0.14 ± 0.02	0.11 ± 0.04	0.05
	C24:6n-3	0.19 ± 0.03	0.10 ± 0.04	
Unsat. Index[d]		292.5 ± 16.8	263.6 ± 12.8	

Values given are means ± S.E.M. For each n 10 retinas were pooled. No significant effects of diet were identified in saturated and monounsaturated fatty acids. [a]n-6, n-6 long chain fatty acids; [b]n-3, n-3 long chain fatty acids; [c]VLCFA, very long chain fatty acids; [d]Unsat. Index, Unsaturated index (number of double bond x % of each fatty acids).

Phosphatidylethanolamine

The major fatty acid in phosphatidylethanolamine of the ROS was C22:6n-3 (28-40% w/w). Phosphatidylethanolamine contained very long chain fatty acids of only 24 carbon chain length with 4, 5 and 6 double bonds as a minor component (Table IV-3). Feeding a high n-3 fatty acid diet increased C22:5n-3 in the ROS from diabetic animals and increased C22:6n-3 in control and total n-3 fatty acids compared to animals fed a low n-3 fatty acid diet. The high n-3 fatty acid diet reduced the level of C20:4n-6 in control and C24:4n-6 in diabetic animals, and C22:4n-6 and C22:5n-6 in both control and diabetic animals. Feeding a high n-3 fatty acid diet decreased the total n-6 level (Table IV-3).

Phosphatidylserine

Very long chain fatty acids composed of C24:4n-6, C24:5n-3 and C24:6n-3 were a major component on phosphatidylserine compared to other phospholipids (Table IV-4).

Table IV-4. Effect of diet on long and very long chain fatty acid composition of phosphatidylserine in rod outer segment.

Diet		High n-3 (n=3)	Low n-3 (n=3)	Significance level (p<)
Fatty acids	(% w/w)			
n-6[a]	C18:2	0.34 ± 0.15	1.77 ± 0.99	
	C20:3	0.07 ± 0.03	0.03 ± 0.03	
	C20:4	5.27 ± 0.50	4.94 ± 1.09	
	C22:4	1.97 ± 0.06	3.57 ± 1.16	0.05
	C22:5	0.27 ± 0.00	2.90 ± 0.91	0.001
n-3[b]	C20:5	0.18 ± 0.02	0.16 ± 0.10	
	C22:5	2.13 ± 0.12	0.91 ± 0.25	0.001
	C22:6	49.45 ± 1.93	41.33 ± 7.70	
VLCFA[c]	C24:4n-6	1.39 ± 0.73	2.72 ± 0.36	0.05
	C24:5n-3	1.66 ± 0.09	1.06 ± 0.42	0.05
	C24:6n-3	1.72 ± 0.07	1.00 ± 0.59	
Unsat. Index[d]		367.5 ± 7.0	337.9 ± 50.0	

Values given are means ± S.E.M. For each n 20 retinas were pooled. No significant effects of diet were identified in saturated and monounsaturated fatty acids. [a]n-6, n-6 long chain fatty acids; [b]n-3, n-3 long chain fatty acids; [c]VLCFA, very long chain fatty acids; [d]Unsat. Index, Unsaturated index (number of double bond x % of each fatty acids).

Feeding a high n-3 fatty acid diet significantly increased membrane content of C22:5n-3 in control and diabetic animals, and C24:5n-3 in diabetic animals. Feeding this high n-3 fatty acid diet decreased the level of C22:4n-6 in diabetic animals and C22:5n-6, C24:6n-6 and total n-6 fatty acids in both control and diabetic animals. Although the ROS content of C22:6n-3 was not affected by diet, the level of C22:6n-3 in phosphatidylserine was the highest among the phospholipid fractions examined.

Phosphatidylinositol

Feeding a high n-3 fatty acid diet significantly increased the levels of C22:5n-3 and C22:6n-3 while decreasing C22:5n-6 compared to feeding a low n-3 fatty acid diet (Table IV-5). Arachidonic acid (C20:4n-6), the major fatty acid in this phospholipid, was not affected by either diet treatment or the diabetic state.

Table IV-5. Effect of diet on long and very long chain fatty acid composition of phosphatidylinositol in rod outer segment.

Diet		High n-3 (n=3)	Low n-3 (n=3)	Significance level (p<)
Fatty acids	(% w/w)			
n-6[a]	C18:2	0.58 ± 0.20	1.11 ± 0.39	
	C20:3	0.16 ± 0.02	0.13 ± 0.07	
	C20:4	33.86 ± 0.33	30.16 ± 4.71	
	C22:4	0.48 ± 0.03	1.35 ± 0.56	
	C22:5	0.09 ± 0.05	0.61 ± 0.07	0.01
n-3[b]	C20:5	0.93 ± 0.00	0.98 ± 0.80	
	C22:5	0.41 ± 0.04	0.12 ± 0.06	0.01
	C22:6	10.22 ± 1.13	5.72 ± 0.90	0.05
VLCFA[c]	C24:4n-6	0.12 ± 0.07	0.24 ± 0.12	
	C24:5n-3	0.22 ± 0.03	0.14 ± 0.14	
	C24:6n-3	0.18 ± 0.05	0.08 ± 0.07	
Unsat. Index[d]		216.9 ± 16.8	185.8 ± 14.8	

Values given are means ± S.E.M. For each n 20 retinas were pooled. [a]n-6, n-6 long chain fatty acids; [b]n-3, n-3 long chain fatty acids; [c]VLCFA, very long chain fatty acids; [d]Unsat. Index, Unsaturated index (number of double bond x % of each fatty acids).

Effect of Diabetic State on Fatty Acid Composition of Rod Outer segment phospholipids

In phosphatidylcholine the diabetic state altered the n-6 fatty acid level in animals fed the low n-3 fatty acid diet (Figure IV-3). The level of C22:5n-6 was higher whereas C26:4n-6 and C28:4n-6 were significantly lower in the diabetic state. In phosphatidylethanolamine the diabetic state increased the level of C22:5n-6, 2C2:6n-3 and total n-3 fatty acids while decreasing C20:4n-6 and total n-6 fatty acids in ROS of animals fed the low n-3 fatty acid diet. The n-6/n-3 ratio was significantly lower in diabetic animals. In phosphatidylserine the diabetic state was characterized by increased levels of C22:5n-6 in animals fed a low n-3 fatty acid diet. Other differences in the fatty acid composition of ROS phospholipids between control and diabetic animals were not found.

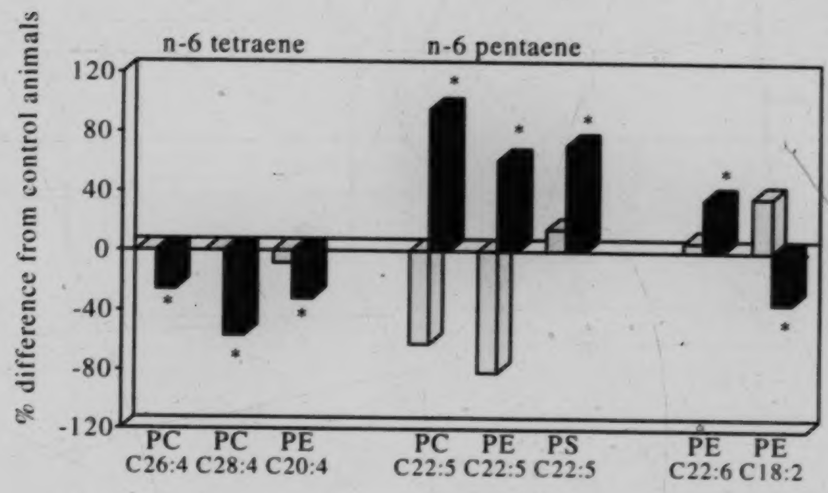

Figure IV-3. Effect of diabetic state on fatty acid composition of phospholipids in rod outer segment. Significant differences (*, $p<0.05$), compared to control animals, were found in diabetic animals fed low n-3 fatty acid diet in all phospholipids. The fatty acid composition (mean ± S.E.M.) for diabetic animals fed the high or low n-3 diet, respectively, is as follows: C26:4 in PC, n.d., 0.20 ± 0.02; C28:4 in PC, n.d., 0.03 ± 0.01; C20:4 in PE, 7.66 ± 0.24, 8.47 ± 0.22; C22:5 in PC, 0.06 ± 0.01, 1.66 ± 0.14; C22:5 in PE, 0.22 ± 0.04, 3.30 ± 0.28; C22:5 in PS, 0.31 ± 0.01, 48 ± 0.53; C22:6 in PE, 42.17 ± 2.31, 38.43 ± 2.42; C18:2 in PE, 0.97 ± 0.18, 0.81 ± 0.07. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. □, high n-3 diet; ■, low n-3 diet.

DISCUSSION

Relationship between Diet and Rod Outer Segment Fatty Acid Composition

In many studies of dietary modulation of n-3 fatty acids in various membrane phospholipid components of nervous tissue (brain and retina) (Anderson et al., 1989; Connor et al., 1990; Neuringer et al., 1986; Philbrick et al., 1987), feeding n-3 fatty acids was compared to feeding n-3 fatty acid deficient animals in an n-6/n-3 fatty acid unbalanced diet. Within physiological intake levels, feeding an increased n-3 fatty acid level in the diet with a relatively constant level of saturated and n-6 fatty acid significantly increased membrane content of total n-3 fatty acids in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. This increase is mainly attributed to

C22:5n-3 and C22:6n-3 with concomitant reductions in C22:4n-6 and C22:5n-6 leading to changes in the n-6/n-3 fatty acid balance in the photoreceptor membranes. These data suggest that retinal tissues may be capable of replacing the n-6 with n-3 fatty acids, demonstrating that ROS is in a dynamic state and undergoes significant membrane fatty acid turnover. This finding is consistent with studies by Hargreaves and Clandinin (1987a & 1987b) who found a strong correlation between dietary fat content of n-6 and n-3 fatty acids and membrane content of long-chain n-6 and n-3 fatty acids in brain microsomal and synaptosomal membranes. This turnover could result from fatty acid incorporation during phospholipid synthesis or by direct substitution of n-6 for n-3 fatty acid by phospholipid deacylation - reacylation. Both of these metabolic pathways are active in the retina and ROS (Bazan & Scott, 1990; Swartz & Mitchell, 1970; Zimmerman & Keys, 1988 & 1989) and might be important for maintaining appropriate membrane components in specialized cells and be responsible for supplying components for renewal of the ROS membrane.

The compensatory replacement of docosahexaenoic acid by docosapentaenoic acid in all phospholipids in the photoreceptor membrane from animals fed the low n-3 fatty acid diet may be relevant to studies of n-3 fatty acid deficiency. Several studies report functional deficits in vision such as vision loss, abnormal electroretinogram in rats, monkeys and human infants fed n-3 deficient diets (Neuringer & Connor, 1986; Philbrick et al., 1987; Uauy et al., 1990 & 1992). The increase in C22:6n-3 induced by diet may provide insight into beneficial effects of a high n-3 diet in retinal disease such as retinitis pigmentosa where plasma levels of C22:6n-3 are low (McLachlan et al., 1990; Newsome et al., 1988). The type of change observed in the fatty acyl tail composition of phospholipids in the present study could affect function of the retina and have potential relationships to clinical disorders.

Changes in Very Long Chain Fatty Acids

The present study is the first to demonstrate dietary responsiveness of very long chain fatty acids in visual cells. Among phospholipids in the ROS, only phosphatidycholine contains very long chain fatty acids up to 34 carbons with 4, 5 and 6 double bonds. These observations are consistent with previous reports for bovine retina (Aveldano & Sprecher, 1987) and brain in humans and rats (Poulos et al., 1986b; Robinson et al., 1990a). Other phospholipids measured contained a maximum of 24 carbon atoms in fatty acids with 4, 5 and 6 double bonds of the n-6 and n-3 series. These observations suggest that different species of a specific phospholipid in the same membrane may exert a specific functional role yet to be defined.

Increased n-3 fatty acid content in the diet significantly reduced the content of all n-6 tetraenoic very long chain fatty acids in phosphatidylcholine with a concomitant increase in C24:5n-3 and C26:5n-3 in the ROS. The level of C24:5n-3 was also increased with decreasing C24:4n-6 in phosphatidylethanolamine and phosphatidylserine. Because the function, proportions or properties of individual very long chain fatty acids in most membrane phospholipids is unknown, the precise implication of these findings is not known. The ROS is the major site of photon absorption. These diet fat-induced changes in of very long chain fatty acids may influence different properties such as cell membrane physical properties and rhodopsin orientation in the membrane and thus altering the functional role of rhodopsin in this specialized membrane.

At present, no study has examined why only phosphatidylcholine contains very long chain fatty acids. During *de novo* synthesis of phospholipid, phosphatidylcholine is a favoured substrate for C22:6n-3 incorporation (Bazan et al., 1986a). Aveldano (1987) found that metabolic activity is devoted to synthesis of very long chain fatty acids and that these fatty acids are made by successive elongation of precursor C20 and C22 polyenes. Shorter chain precursors are derived from C18:2n-6 or C18:3n-3 supplied by the diet. The results of the present study indicate that dietary fat alters the short chain precursor availability to increase (or decrease) the synthesis of very long chain fatty acids and also suggest that synthesis of very long chain fatty acids occurs in the retina and ROS.

Relationship between Diabetic State and Change in Fatty acid Composition

In the present study, the effect of a short period of diabetes on change in membrane composition was confined to a few fatty acids present in small levels. These findings are not consistent with previous reports that synthesis of polyenoic fatty acid is depressed in the diabetic state resulting in reduced levels of arachidonic acid and docosahexaenoic acid in the retina (Futterman et al., 1968b & 1969). This inconsistency might be explained by the diet fed in the present study containing a higher amount of essential fatty acid compared to the commercial rat diet fed by these authors (Futterman et al., 1968b & 1969) or if the high fat diet fed in the present study provided enough essential fatty acid to prevent a response to insulin-induced change in fatty acid composition in the diabetic state. The retina also appears to conserve polyunsaturated fatty acids by rapidly taking up C22:6n-3 from the blood (Scott & Bazan, 1989) or by recycling C22:6n-3 between retinal pigment epithelium and the ROS (Stinson et al., 1991). In the diabetic state the retina may retain essential fatty acid to be resistant to the disease.

The findings of the present study provide the first experimental evidence for diet-induced alteration of long and very long chain fatty acid constituents of photoreceptor cell membrane phospholipid. These effects on membrane include significant alterations in long and very long chain fatty acids in phospholipids as well as a reciprocal replacement of C22:5n-6 by C22:6n-3 and n-6 tetraenoic VLCFA by n-3 pentaenoic very long chain fatty acids. It is concluded that since these very long chain fatty acids of C24 to C34 carbon chain length are not of direct dietary origin they must be synthesized from shorter chain essential fatty acid precursors present in the diet fed. The functional roles for very long chain n-6 and n-3 fatty acids in the retina remain to be determined.

Comparisons Between Retina and Rod Outer Segment

Although this section is not part of the paper published, an earlier experiment was carried out on the entire retina with the same protocol used in this chapter. The concentration of total fatty acids was compared to observe differences between the entire retina and rod outer segments. Diet and disease effects on the entire retinal fatty acid profile are similar to those effects observed on the rod outer segment. Sample differences were identified with three-way analysis of variance procedures for all phospholipids (see Appendix, Figure A-5 to A-7).

Phosphatidylcholine, Phosphatidylethanolamine and Phosphatidylserine

The levels of total n-6 and n-3 fatty acids, as well as, n-6 and n-3 very long chain fatty acids were significantly higher in the rod outer segment compared to the whole retina in phosphatidylcholine and phosphatidylserine (see Appendix, Figure A-5; A-7). The ratio of n-6 to n-3 fatty acid was significantly higher in the retina in phosphatidylcholine. Saturated fatty acids were significantly higher in the retina in all three phospholipids (see Appendix, Figure A-5 to A-7). The retina also exhibited a high level of monounsaturated fatty acids in phosphatidylcholine.

Chapter V.

Dietary Fatty Acids Modulate the Content of Long and Very Long Chain Fatty Acids (C24-C36), Rhodopsin Content and Rhodopsin Phosphorylation in Rat Photoreceptor Cells after Light Exposure.

INTRODUCTION

Many studies have confirmed dietary modulation of n-3 fatty acids in the various membrane phospholipid components of nervous tissue such as brain and retina in the chick (Anderson et al., 1990), monkey (Neuringer et al., 1986; Lin et al., 1994; Connor et al., 1990) and rat (Philbrick et al., 1987). These diet induced modulations of docosahexaenoic acid content in the membrane alter retinal functions when measured by electroretinogram visual acuity (Neuringer et al., 1986; Pawlosky et al., 1997; Weisinger et al., 1996a & 1996b). In most of these studies, fish oil was used as the only source of dietary fat and experiments were performed on n-3 fatty acid deficient animals by feeding an unbalanced n-6/n-3 fatty acid diet. Studies investigating the effect of dietary fat, within practical fat intake levels, on individual phospholipid classes in visual cells in normally growing animals are limited.

The role of dietary fat in determining the composition and content of very long chain fatty acids has not been studied much despite being well established for longer chain fatty acid in visual cells. Very long chain fatty acids comprising carbon chain length up to 36 are highly concentrated, exclusively in phosphatidylcholine of photoreceptors (Aveldano, 1987 & 1988; Chapter IV). The functional role of very long chain fatty acids in retina has not been identified. In the previous study using a balanced n-6/n-3 fatty acid diet, very long chain fatty acids responded to change in dietary fat fed (Chapter IV; Suh et al., 1994). Rhodopsin is firmly embedded in the lipid bilayer in the disk of the rod outer segment and its function is sensitive to specific alteration in membrane phospholipid (Bush et al, 1994; Shichi, 1971).

Animals exposed to light contain a lower level of rhodopsin coupled with shortened rod outer segments length, reduced number of photoreceptors and thinner outer nuclear layer and reduced rhodopsin synthesis (Schremser and Williams, 1995a & 1995b; Organisciak et al., 1996; Penn and Williams, 1986). These changes maybe partly related with docosahexaenoic acid since this fatty acid is reduced by constant illumination and bright cyclic illumination (Wiegand et al., 1986; Penn & Anderson, 1987; Wiegand et al., 1995). These findings imply that altering membrane fatty acid composition by dietary fat may modulate membrane components of photoreceptor cells after light stress.

It is logical to determine if dietary fat and light induced changes in long and very long chain fatty acids alters visual pigment function in the photoreceptor cell of growing animals. Thus, the objective of this study was to determine whether dietary fat containing high or low n-3 fatty acid alters the levels of polyenoic long and very long chain fatty acids in individual phospholipid of the rod outer segment after 48 hour light exposure. In order to relate these changes to visual function, rhodopsin level and rhodopsin phosphorylation were measured in the rod outer segment.

MATERIALS AND METHODS

Animals and Diets

Male weaning Sprague-Dawley rats were housed under cyclic light with 12 hour dark and 12 hour light periods. The intensity of illumination at the front of the cage was 110 lux (1.52 Quantum, μEm^2sec^{-1}) and at the centre 27 lux (0.38 Quantum, μEm^2sec^{-1}) of cool, white fluorescent light. Animals were randomly assigned to two diet groups and fed semipurified diets containing 20% (w/w) fat of either a high (4.8%, w/w) n-3 fatty acid providing C22:6n-3 (3.4%, w/w) or a low (1.2%, w/w) n-3 fatty acid without C22:6n-3. Shark oil was used to supply C22:6n-3 in the high n-3 diet, since it is high in content of C22:6n-3 (22%, w/w) and relatively low in C20:5n-3 (5.5%, w/w) compared to fish oil used previously (Suh et al., 1994). The fat mixture and fatty acid composition of diets fed is presented in Table V-1 and reflect a range of dietary fat intake typical in North America.

Table V-1. Fat mixture and fatty acid composition of experimental diets.

Diet	High n-3	Low n-3
Fat source (g/kg)		
Beef tallow	134.0	152.0
Safflower	33.0	44.0
Linseed oil	-	4.0
Shark oil	36.0	-
Fatty acid (% w/w)		
C14:0	2.8	2.4
C16:0	23.9	22.9
C18:0	46.2	47.9
C18:1n-9+n-7	5.7	6.3
C18:2n-6	11.3	16.3
C18:3n-3	0.1	1.2
C20:4n-6	0.3	-
C20:5n-3	0.9	-
C22:6n-3	3.4	-
ΣSAT[a]	76.1	76.0
ΣMono[b]	7.3	6.5
Σn-6[c]	11.8	16.3
Σn-3[d]	4.8	1.2
P/S ratio[e]	0.22	0.23

Fatty acids with chain length greater than C24 were present less than 0.02% (w/w) in both diets. [a] ΣSAT, sum of saturated fatty acids; [b]ΣMono, sum of mono unsaturated fatty acids; [c] Σn-6, sum of n-6 fatty acids; [d] Σn-3, sum of n-3 fatty acids; [e] P/S ratio, polyunsaturated to saturated fatty acid ratio.

After 6 weeks of feeding half of the rats in each diet group were transferred to polypropylene cages, housed in pairs and exposed to 48 hours of continuous light at the intensity of 1000 lux (12 Quantum, $\mu Em^2 sec^{-1}$). The other half of each diet group were kept in complete darkness as a control. Diets were prepared weekly, kept in the freezer and fed to animals every other day. Body weight was taken weekly and the weight gain from weaning (54.1 ± 4.5 g) to 6 weeks post weaning (374.6 ± 30 g) was 7.2 g per day. No effect of dietary treatment was observed in body weight or weight gain.

Isolation of Retina and Rod Outer Segment (ROS)

Animals were sacrificed by decapitation. All isolations were performed at 4°C. For lipid analysis, retinas were isolated either in dim red light for dark adapted animals or in ambient laboratory light for light exposed groups. For the analysis of rhodopsin, light exposed animals were dark adapted for 1 hour before sacrifice in dim red light. For each sample at least 8 retinas were pooled for the separation of the rod outer segment (ROS) for lipid analysis and 10 retinas for the measurement of rhodopsin content and rhodopsin phosphorylation. The ROS was isolated by discontinuous sucrose gradient centrifugation (Stinson et al., 1991a). Freshly prepared ROS for lipid analysis was used immediately after homogenation with a polytron. The ROS for rhodopsin analysis was resuspended with 0.5 ml of washing buffer, 50 mM Tris-acetate (pH 7.4) containing 5 ml $MgCl_2$ and 0.1 mM EDTA and then stored at -70°C until use. Scanning electron micrographs of retina of either dark adapted or light exposed for 48 hours after feeding a low n-3 fatty acid diet are presented in Figure A-8 (see Appendix).

Lipid Analysis

Lipids were extracted from the ROS (Folch et al., 1957). Individual phospholipids were prepared from the extracts using silica-gel TLC H-plates as described previously (Suh et al., 1994). After scraping the phospholipids, the silica gel containing neutral lipids was also scraped into 25 ml culture tubes and eluted three times with 10 ml of chloroform : methanol (1:1, v/v) containing 1 ppm ethoxyquin. The eluted lipids were dried under nitrogen and redissolved in 0.2 ml chloroform for the further separation of neutral lipids. Extracts were separated on prewashed silica-gel TLC G-plates (20 x 20) developed in the following solvent system:petroleum ether:diethyl ether:acetic acid (80:20:1, by vol). Plates were allowed to dry at room temperature for 2-3 min and visualized with 0.01% (w/v) anilino naphthalene sulfonic acid in water under U.V. light. Each phospholipid and neutral lipid was recovered and fatty acid methyl esters prepared with 14% (w/w) boron trifluoride-methanol reagent (Morrison and Smith, 1964).

Analysis of Fatty Acid Methyl Esters

Fatty acid methyl esters were separated on a polar BPX 70 column (25 m x 0.22 mm I.D.) using a Hewlett Packard 5890 GLC equipped with a Vista DS 654 data system, as described earlier (Suh et al., 1994). With this column, the resolution of n-6 tetraenoic and pentaenoic and n-3 pentaenoic and hexaenoic very long chain fatty acids was more distinct compared to a non polar BP1 column used earlier (Suh et al., 1994) (Figure V-1). Degree of unsaturation and n-series of very long chain fatty acids were confirmed by GC-MS (Suh et al., 1994). The content of very long chain fatty acids obtained was not based on response factors, since standards for these fatty acid are not available.

Rhodopsin Measurement

Rhodopsin content of the ROS was determined from animals of both dietary groups that were either dark adapted or light exposed. Light exposed animals were dark adapted for 1 hour prior to being sacrificed. Rhodopsin was extracted by the method of Fulton et al. (1982) with a slight modification using 1% (w/v) octylglucoside in 0.067 M phosphate buffer (pH 6.7). Octylglucoside is capable of rapidly solublizing the disc membrane while maintaining the structure and function (Stubbs et al., 1976). After extraction, 25 ul of 1 M hydroxylamine at pH 7.0 was added to each tube and then a clear rhodopsin extract was obtained by centrifugation at 14,000 g for 30 min. Each extract was scanned from 270 to 700 nm using a diode-array spectrophotometer (Hewlett Packard 8452A) in the complete darkness and then scanned at 5, 10, 15, 30, 60, 120, 180, 240, 360 and 480 seconds during bleaching with a cool white fluorescent light with the intensity of 1,000 lux (14 Quantum, $\mu Em^2 sec^{-1}$) at a distance of 30 cm from the cuvette. Rhodopsin was quantified by difference spectroscopy at 498 nm absorption before and after bleaching for 480 second using a molar extinction coefficient of 40,000 (Hubbard et al., 1971). The measurement at each time point was used to calculate the kinetics of rhodopsin bleaching.

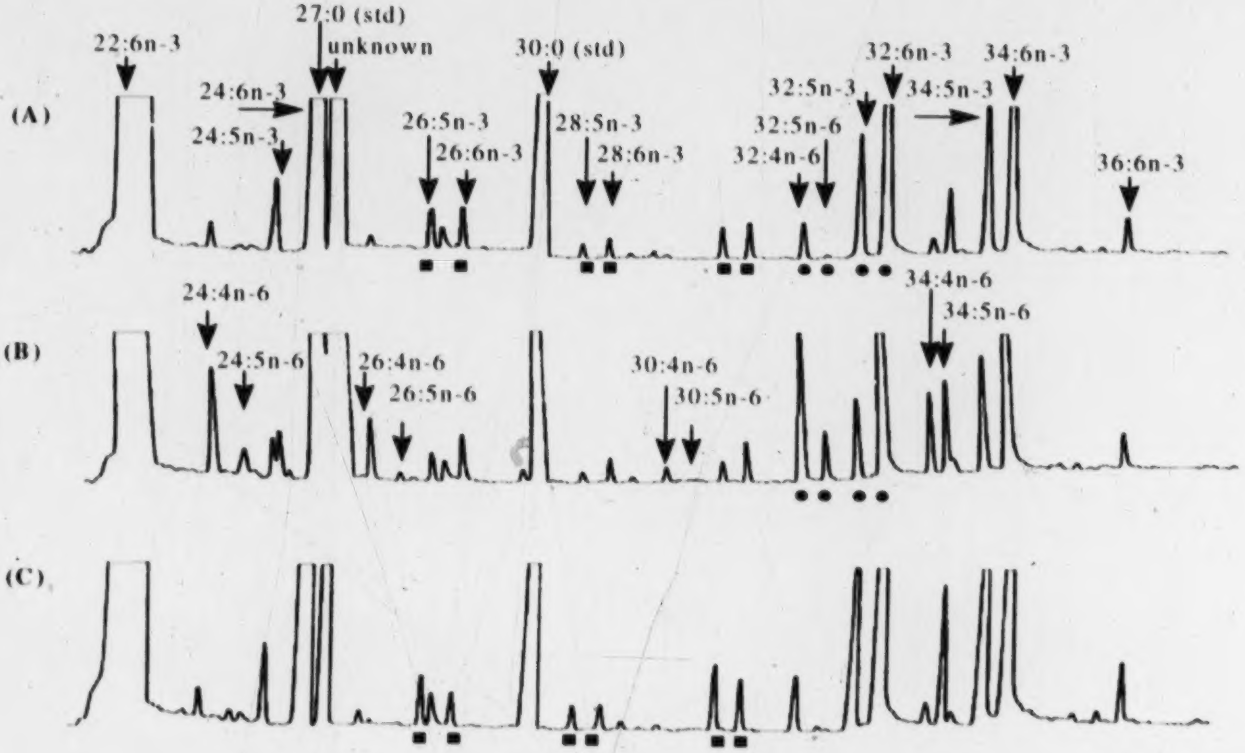

Figure V-1. Typical chromatograms of very long chain fatty acids of phosphatidylcholine in the rod outer segment following diet and light treatment. Although the amount of standard is different in (A) and (B), the proportion of the fatty acids indicated by the circle (●) in C32 indicate an example of the influence of diet treatment. Those indicated by the square (■) in (A) and (C) show the examples of the reflection of light treatment. (A), high n-3 diet/dark; (B), low n-3 diet/dark; (C), high n-3 diet/light.

Rhodopsin phosphorylation *in Vitro*

Rhodopsin phosphorylation was measured by slight modification of the method used by Kuhn & Wilden (1982). Under dim red light, the ROS containing 350 μg protein was homogenized, transferred to 1.5 ml Eppendorf tubes. The homogenates were suspended in a phosphorylating buffer, 100 mM potassium phosphate (pH 7.4) containing 0.1 mM sodium orthovanadate (Na_3VO_4), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in dimethylsulphoxide (DMSO) and 10 mM dithiothreitol (DTT). Three mM [γ-^{32}P]ATP (specific activity 3000 Ci/mmol, New England Nuclear-DuPont, Boston, MA) was added to the mixture to monitor protein phosphorylation and then the mixture was sonicated for 2 min in a sonic water bath (Branson 2200, Branson Ultrasonics Co., Danbury, CT). Before the reaction was initiated by the onset of illumination, the mixture was warmed to 30°C in a water bath for 3 min in the dark to allow temperature equilibration. The light source used was cool, white fluorescence light at 700-800 lux at a distance of 50 cm from the water bath. After incubation for 1 hour the reaction was terminated by addition of 0.4 ml of 45% (w/v) trichloroacetic acid (4°C) and 15 mM ATP. The ROS membrane was coagulated on ice and then recovered by centrifugation for 5 min at 10,000 g in the fume hood. The pellets were washed 3 times with 1.5 ml of 10% (w/v) trichloroacetic acid containing 5 mM H_3PO_4. The final pellets were resuspended in SDS-PAGE sample buffer (Laemmli, 1970).

The radiolabeled solubilized ROS membrane containing 30 μg of protein was loaded and run on a 10 cm linear gradient (7.5% acrylamide) polyacrylamide gel for 2 hours at a constant current at 40 mA at ≈150 voltage. The gel was dried using gel drying film (Promega, Medison, WI). Incorporation of ^{32}Pi was monitored by exposing x-ray film (Kodak X-OMAT AR film) to the gel at -70°C for 1.5 hours. The film was developed by Kodak M35A X-Omat Processors (Rochester, New York). The resulting autoradiograms were analyzed using a scanning densitometery (Ultroscan XL, Bromma, Sweden). The area under the peaks was used to quantitate labeling with ^{32}Pi.

Statistical Methods

The effect of light and diet on the fatty acid composition of phospholipid in the ROS and on the rhodopsin content and rhodopsin phosphorylation was analyzed by two-way analysis of variance with light and diet as the main factors. The n is indicated in each table and figure caption. Duncan's multiple range test (Steel & Torrie, 1990) was used for comparison of the main factors. All data is expressed as mean ± standard error of the mean (S.E.M.).

RESULTS

Effects of Dietary Fat and Light on Long and Very Long Chain Fatty Acid Composition of ROS Phospholipids

In the present study, both diet and light significantly altered the fatty acid composition of the major phospholipids of the ROS in rat retina. Feeding a high C22:6n-3 diet increased the level of C22:6n-3 in phosphatidylethanolamine and phosphatidylserine in both dark or light exposed animals (Table V-2). This diet also increased the level of C22:6n-3 in phosphatidylinositol of light adapted animals. Concomitantly the level of C20:4n-6 decreased in all major phospholipids except phosphatidylinositol of dark adapted animals after feeding a high n-3 fatty acid diet containing C22:6n-3.

After 48 hours of light exposure *in vivo*, retinas of animals fed the high n-3 fatty acid diet lost C22:6n-3 in phosphatidylcholine (20.3%) and phosphatidylserine (5.1%) (Table V-2). Animals fed a low n-3 fatty acid diet containing no C22:6n-3 exhibited a significant decrease in C20:4n-6 in phosphatidylethanolamine and C22:6n-3 in phosphatidylserine after light exposure. The level of C20:4n-6 in phosphatidylinositol and C22:6n-3 in phosphatidylethanolamine increased after light exposure.

Very long chain fatty acids containing carbon chain length up to C36 in phosphatidylcholine and C26 in phosphatidylserine were also influenced by both diet and light treatment (Table V-3; Figure V-1; V-2; V-4). For example, in phosphatidylcholine of dark adapted animals, feeding a high n-3 fatty acid diet containing C22:6n-3 increased the level of n-3 pentaenoic very long chain fatty acid of C24, C26, C30, C32 and

Table V-2. Effect of dietary fat and light on the level of C20:4n-6 and C22:6n-3 phospholipids in the rod outer segment.

Diet	High n-3		Low n-3		Significant effects	
Fatty acids (% w/w)	Dark	Light	Dark	Light	Light	Diet
Phosphatidylcholine						
C20:4n-6	3.9 ± 0.2^{b}	4.2 ± 0.1^{b}	5.0 ± 0.2^{a}	5.1 ± 0.2^{a}		***
C22:6n-3	32.5 ± 2.1^{a}	25.9 ± 1.1^{b}	27.2 ± 1.9^{ab}	24.8 ± 2.4^{b}	*	
Phosphatidylethanolamine						
C20:4n-6	5.9 ± 0.3^{c}	5.8 ± 0.2^{c}	7.6 ± 0.4^{a}	6.7 ± 0.1^{b}		***
C22:6n-3	57.1 ± 1.3^{a}	58.1 ± 0.5^{a}	47.5 ± 0.9^{c}	53.1 ± 0.4^{b}	**	***
Phosphatidylserine						
C20:4n-6	2.5 ± 0.2^{b}	2.5 ± 0.1^{b}	3.1 ± 0.1^{a}	2.9 ± 0.1^{a}		***
C22:6n-3	63.1 ± 0.9^{a}	59.9 ± 0.4^{b}	55.7 ± 0.6^{c}	52.4 ± 0.5^{d}	***	***
Phosphatidylinositol						
C20:4n-6	37.3 ± 0.9^{b}	38.7 ± 0.8^{b}	39.5 ± 1.1^{b}	44.6 ± 0.7^{a}	**	**
C22:6n-3	12.5 ± 1.0^{b}	15.8 ± 0.5^{a}	11.7 ± 1.0^{b}	12.2 ± 0.5^{b}	*	*

Values given are means (n=6) ± S.E.M. For each n 8 retinas were pooled, except phosphatidylinositol (n=3) 16 retinas pooled. Significant effects of light and diet were analyzed by two-way analysis of variance. Values without a common superscript are significantly different: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Significant interactions were identified only for C22:6n-3 in phosphatidylethanolamine.

C34 carbons in chain length. This diet also decreased most of the n-6 tetraenoic very long chain fatty acid except for C30 and C36 and decreased n-6 pentaenoic very long chain fatty acid of C24, C26 and C34 carbons in chain length (Table V-3). Hexaenoic n-3 very long chain fatty acids were not influenced by a high n-3 fatty acid diet. The high n-3 fatty acid diet increased total n-3 pentaenoic very long chain fatty acids in phosphatidylcholine, while decreasing the n-6 tetra and pentaenoic very long chain fatty acids (Figure V-3). In phosphatidylserine, the content of n-3 pentaenoic very long chain fatty acid of C24 and C26 and hexaenoic C26 fatty acids were increased by feeding a high n-3 fatty acid diet. Whereas this diet decreased the level of n-6 tetraenes of C24 and pentaenes of C24 and C26 carbons in chain length (Table V-3).

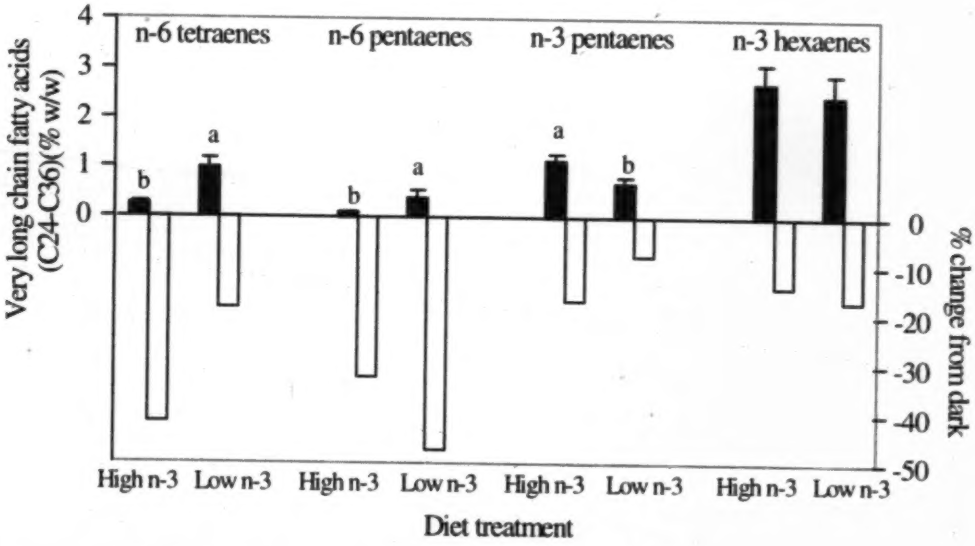

Figure V-2. Effect of dietary fat and light on the level of total n-6 tetraenoic and pentaenoic and total n-3 pentaenoic and hexaenoic very long chain fatty acid (C24 - C36) present in phosphatidylcholine of the rod outer segment. The values for the dark adapted group are the mean (n=6) ± S.E.M. Eight retinas were pooled for each n. The value of % change from dark by light treatment was taken from the total mean of each group. Values without a common letter within a group of fatty acids indicate a significant effect of diet, $p<0.01$. N-6 and n-3 very long chain fatty acids were lost by light exposure but not significantly different from dark adapted group. ■, dark; □, light.

In light exposed animals, total n-6 tetraenoic and pentaenoic very long chain fatty acids decreased in animals fed either high (46.0% and 32.5% respectively) or low n-3 fatty acid (18.4% and 47.2%, respectively) (Figure V-2). Total n-3 pentaenoic and

Table V-3. Effect of dietary fat on the level of very long chain fatty acid composition of phosphatidylcholine (C24-C36) and phosphatidylserine (C24-C26) in the rod outer segment of dark adapted rats.

Diet	Phosphatidylcholine		Phosphatidylserine	
Fatty acids (% w/w)	High n-3	Low n-3	High n-3	Low n-3
n-6 tetraenoic VLCFA^{a}				
C24:4	$0.06 \pm 0.01^{***}$	0.15 ± 0.01	$0.43 \pm 0.01^{***}$	1.73 ± 0.18
C26:4	$0.02 \pm 0.00^{***}$	0.06 ± 0.01	0.05 ± 0.02	0.03 ± 0.02
C28:4	$0.00 \pm 0.00^{***}$	0.02 ± 0.00		
C30:4	0.02 ± 0.01	0.03 ± 0.00		
C32:4	$0.11 \pm 0.03^{***}$	0.29 ± 0.04		
C34:4	$0.04 \pm 0.01^{***}$	0.21 ± 0.05		
n-6 pentaenoic VLCFA				
C24:5	$0.02 \pm 0.00^{***}$	0.05 ± 0.01	$0.09 \pm 0.02^{***}$	0.29 ± 0.03
C26:5	$0.00 \pm 0.00^{**}$	0.02 ± 0.00	$0.00 \pm 0.00^{*}$	0.03 ± 0.02
C28:5	0.00 ± 0.00	0.00 ± 0.00		
C30:5	0.01 ± 0.00	0.01 ± 0.01		
C32:5	$0.02 \pm 0.01^{***}$	0.14 ± 0.05		
C34:5	0.03 ± 0.00	0.10 ± 0.04		
n-3 pentaenoic VLCFA				
C24:5	$0.15 \pm 0.02^{**}$	0.08 ± 0.01	$1.59 \pm 0.08^{***}$	0.86 ± 0.08
C26:5	$0.08 \pm 0.01^{**}$	0.04 ± 0.00	$0.06 \pm 0.01^{**}$	0.03 ± 0.01
C28:5	0.03 ± 0.00	0.02 ± 0.00		
C30:5	$0.07 \pm 0.01^{**}$	0.04 ± 0.01		
C32:5	$0.31 \pm 0.05^{**}$	0.17 ± 0.03		
C34:5	$0.49 \pm 0.06^{**}$	0.32 ± 0.08		
n-3 hexaenoic VLCFA				
C24:6	-	-	2.01 ± 0.44	1.80 ± 0.21
C26:6	0.08 ± 0.01	0.08 ± 0.01	$0.17 \pm 0.01^{*}$	0.12 ± 0.03
C28:6	0.06 ± 0.01	0.04 ± 0.01		
C30:6	0.07 ± 0.01	0.07 ± 0.01		
C32:6	1.32 ± 0.21	1.10 ± 0.17		
C34:6	1.10 ± 0.14	1.00 ± 0.18		
C36:6	0.09 ± 0.01	0.08 ± 0.01		

Values given are means (n=6) ± S.E.M. For each n 8 retinas were pooled. Superscript* indicates the significant difference between diets within each phospholipid: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. ^{a}VLCFA, very long chain fatty acids. C24:6n-3 of phosphatidylcholine was detected but not expressed, because its resolution time was almost the same as a standard, C27:0.

hexaenoic very long chain fatty acids also decreased in both the high (16.9% and 14.3%) and the low (7.8% and 17.0%) n-3 fatty acid groups. The overall level of n-6 and n-3 very long chain fatty acids was not significantly different from the dark adapted group. The loss of n-6 tetraenoic and pentaenoic very long chain fatty acid after light treatment was slightly more than n-3 very long chain fatty acids (Figure V-2).

Effect of Dietary Fat and Light on fatty Acid Composition of Free Fatty Acid and Triglyceride

To identify the fate of C20:4n-6 and C22:6n-3 lost from phospholipids after light treatment, free fatty acid and triglyceride fractions were also analyzed (Figure V-3). In both lipid classes, diet and light treatment greatly influenced the level of C20:4n-6 and C22:6n-3. In the free fatty acid fraction, feeding the high n-3 fatty acid diet significantly increased C22:6n-3 in both dark and light adapted animals. In the triglyceride fraction, the level of C22:6n-3 was reduced in the dark adapted group with a concomitant

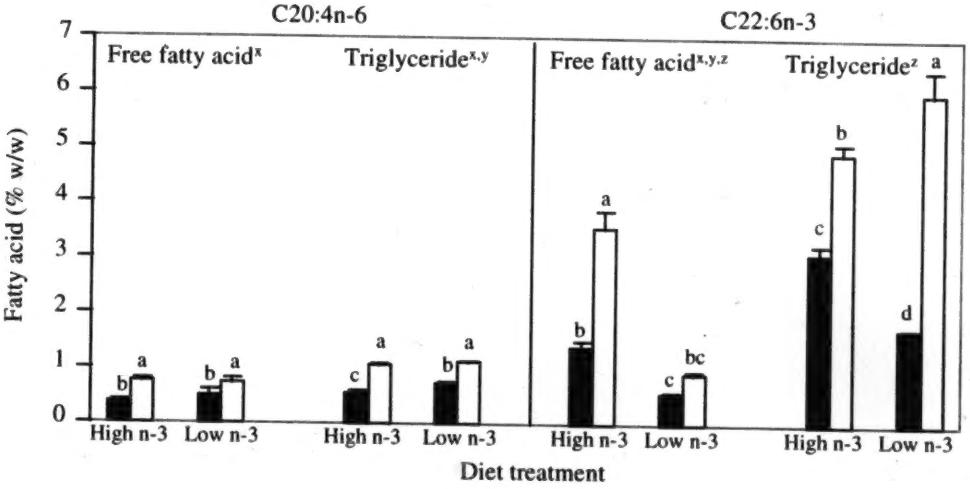

Figure V-3. Effect of dietary fat and light on the level of C20:4n-6 and C22:6n-3 in free fatty acid and triglyceride components of the rod outer segment. The values are the mean (n=3) ± S.E.M. Sixteen retinas were pooled for each n. Values without a common letter within a lipid class were significantly different, $p<0.05$. Superscripts indicate as follows: significant effect of light (^{x}, $p<0.003$); significant effect of diet (^{y}, $p<0.02$); significant effect of interaction (^{z}, $p<0.003$). ■, dark; ☐, light.

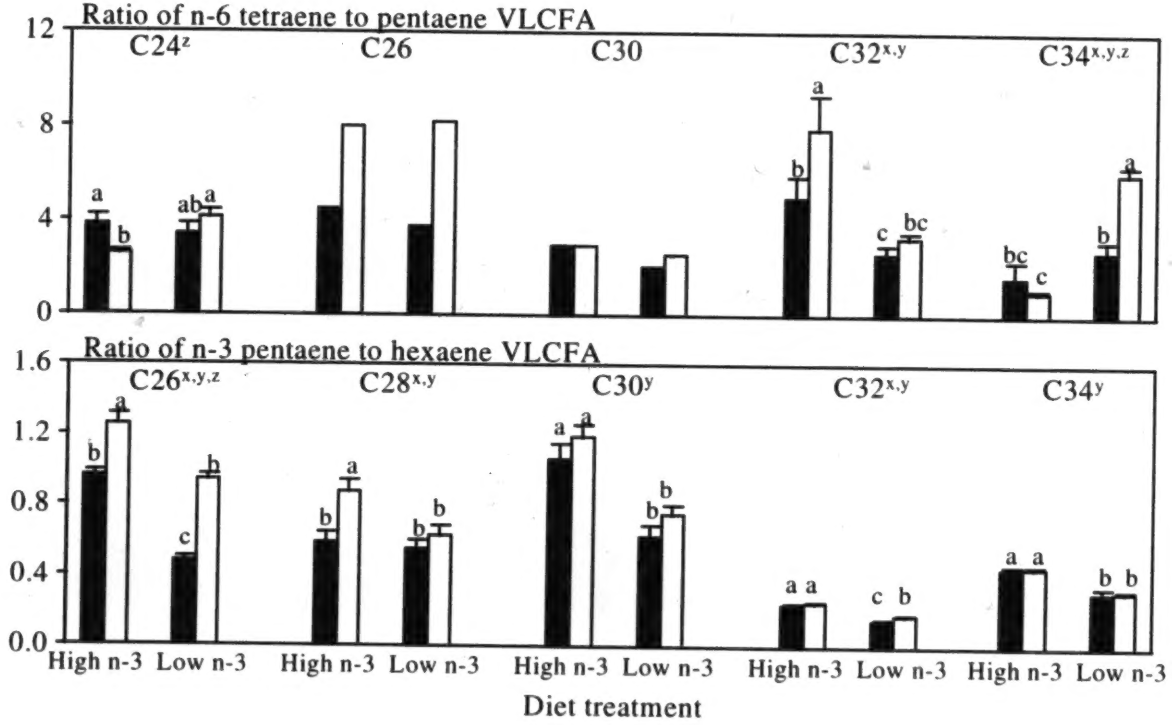

Figure V-4. Effect of dietary fat and light on the ratio of n-6 tetraene to pentaene and of n-3 pentaene to hexaene of very long chain fatty acid present in phosphatidylcholine of the rod outer segment. The values are the mean (n=6) ± S.E.M. Eight retinas were pooled for each n. Values without a common letter within a fatty acid are significantly different, $p<0.05$. Superscripts indicate as follows: significant effect of light (^{x}, $p<0.02$); significant effect of diet (^{y}, $p<0.0001$); significant interaction (^{z}, $p<0.05$). Where error bars are not indicated, the ratio was taken from the total mean of each group since not all of these fatty acids were detectable in each sample. VLCFA, very long chain fatty acid. ■, dark; □, light.

reduction in C20:4n-6 occurring after feeding a high n-3 fatty acid diet (Figure V-3). Animals exposed to light significantly increased the level of both C20:4n-6 and C22:6n-3 in the free fatty acid and triglyceride fractions in both dietary groups. This suggests that both free fatty acid and triglyceride are the reservoirs for both C20:4n-6 and C22:6n-3 to enable recycling of essential fatty acid after light exposure.

Effect of Dietary Fat and Light on the Ratio of n-6 Tetraene to Pentaene and n-3 Pentaene to Hexane of Very Long Chain Fatty Acids in Phosphatidylcholine

The effect of diet and light treatment on the ratio of n-6 tetrene to pentaene and of n-3 pentaene to hexaene very long chain fatty acids were examined on phosphatidylcholine of the ROS (Figure V-4). Feeding a diet high in n-3 fatty acid exhibited the highest ratio of n-3 pentaenoic to hexaenoic very long chain fatty acid of C26 to C34 compared to that of

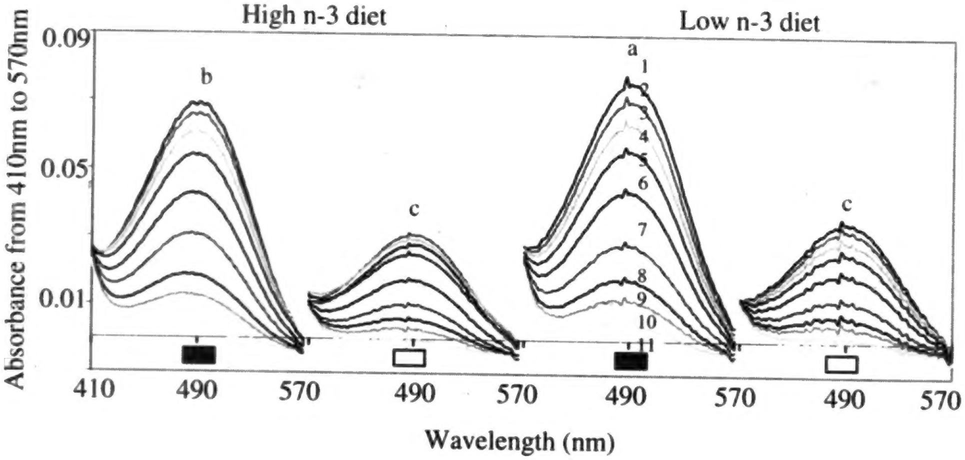

Figure V-5. The absorption spectra of rhodopsin of rod outer segments following dietary fat and light treatment. Each spectra is the mean (n=6) for each group. Eight retinas were pooled for each n. The rhodopsin content was calculated by the change in absorption at 498nm before and after bleaching for 480 seconds. Spectra without a common letter are significantly different, $p<0.05$. Significant effects of light and diet are identified. The rhodopsin level (nmole/mg protein, mean ± S.E.M) from left to right is as follows: 6.1 ± 0.2, 3.0 ± 0.3, 7.5 ± 0.5, 3.1 ± 0.1. Curve 1 is the absorption spectrum of rhodopsin before bleaching. The spectra 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 were scanned at 5, 10, 15, 30, 60, 120, 180, 240, 360 and 480 seconds after bleaching start. These time points were used to calculate the kinetics of rhodopsin disappearance. ■, dark; □, light.

the ROS from animals fed the low n-3 fatty acid diet. This implies that pentaenoic very long chain fatty acids respond more to a diet high in n-3 fatty acid than hexaenes. After animals were exposed to light, the ratio of n-6 tetraene to pentaene very long chain fatty acid in C32 significantly increased in the high n-3 fatty acid group. The ratio of n-6 tetraene to pentaene very long chain fatty acid ratio in C34 also increased following light exposure in the low n-3 fatty acid diet group (Figure V-4). The ratio of n-3 pentaenes to hexaenes increased in C26 fatty acids in both dietary groups. In C28 fatty acids from animals fed the high n-3 diet and C32 fatty acids of the low n-3 diet group, the ratio of n-3 pentaenes to hexaenes also increased This implies that light exposure activates n-6 tetraenes and n-3 pentaenes more than the other homologues.

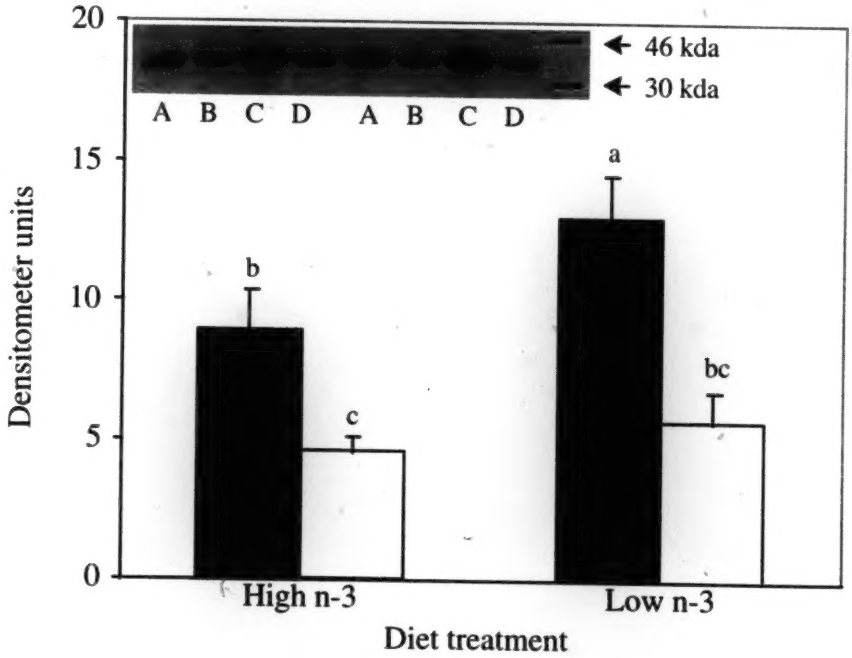

Figure V-6. Effect of dietary fat and light on rhodopsin phosphorylation in the rod outer segment. The values are the mean (n=6) ± S.E.M. Eight retinas were pooled for each n. Values without a common letter are significantly different, $p<0.05$. Significant effects of light and diet were identified. Autoradiograph inset in duplicate: A, high n-3 diet/dark; B, high n-3 diet/light; C, low n-3 diet/dark; D, low n-3 diet/light. Each lane represents 30 µg protein of rod outer segment. ■, dark; □, light.

Effect of Dietary Fat and Light on the Rhodopsin Content

Rhodopsin content of the ROS was determined to identify the relationship between dietary fat and retinal function. The spectrum of the photopigment (Figure V-5) reflects the level of rhodopsin *in situ*. Animals fed a low n-3 fatty acid diet had higher rhodopsin content in dark maintained ROS, than animals fed the high n-3 fatty acid (7.5 nmole/mg protein and 6.1 nmole/mg protein respectively). Significant loss of rhodopsin was found in animals initially exposed to light *in vivo* (Figure V-5, Figure V-7). The loss from the low n-3 fatty acid group was slightly higher than for animals fed a high n-3 fatty acid diet (59% and 51%, respectively, Figure V-7).

Effect of Dietary Fat and Light on the Rhodopsin Phosphorylation

Rhodopsin phosphorylation in the ROS was analyzed *in vitro* to identify the effect of dietary fat on retinal function. Rhodopsin phosphorylation rate was altered by 1 hour of incubation in continuous white light by both the dietary fats fed and by the light

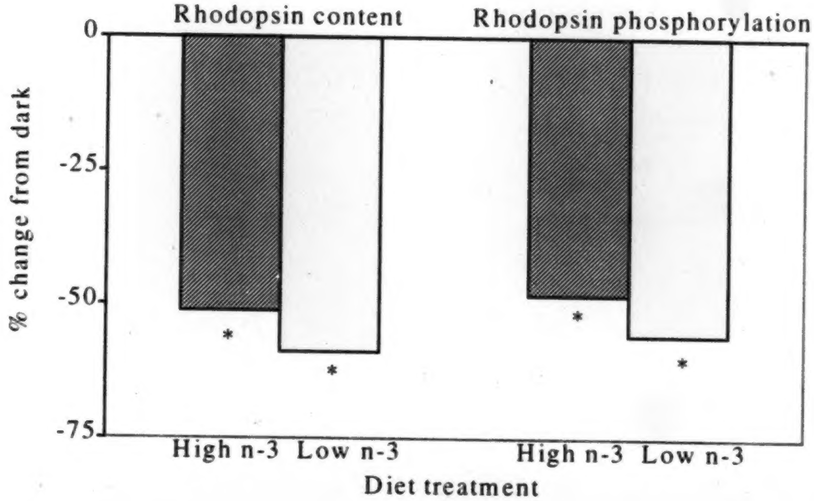

Figure V-7. Effect of light on the change in the rhodopsin content and rhodopsin phosphorylation for animals fed the high versus low n-3 fatty acid treatment. The value of % change from dark was from the total mean of each group and statistical analysis was carried out on the data in Figure V-5 and Figure V-6. Significant loss (* $p<0.0001$) by light treatment was found in rhodopsin content and rhodopsin phosphorylation for animals of both diets.

treatment. Compared to light exposed animals, dark adapted animals exhibited significantly higher rhodopsin phosphorylation (Figure V-6). Rhodopsin in the ROS of animals fed a low n-3 fatty acid exhibited higher phosphorylation than observed for animals fed the high n-3 fatty acid diet. However, once exposed to the light *in vivo*, the rhodopsin from the low n-3 fatty acid diet group phosphorylated only 44% of dark adapted rhodopsin compared to 52% in animals fed the high n-3 fatty acid diet (Figure V-7).

DISCUSSION

Dietary Fat and Long Chain Fatty Acids

In normal growing rats, feeding nutritionally adequate diets with a high n-3 or low n-3 fatty acid content produced considerable modification of the fatty acid composition in photoreceptor cell membrane phospholipids. Increased n-3 fatty acid level with addition of C22:6n-3 in the diet with a fixed level of saturated and monounsaturated fatty acid significantly increased membrane content of docosahexaenoic acid in phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. These increases were associated with reductions in C20:4n-6 resulting in higher n-3 fatty acid in photoreceptor membranes. This finding supports the previous study that the neuronal cell is capable of replacing n-6 with n-3 fatty acids (Hargreaves & Clandinin, 1987a & 1987b; Suh et al., 1994). The replacement of n-6 with n-3 fatty acid is more obvious in animals deficient in n-3 fatty acids (Connor et al., 1990 & 1991; Neuringer & Connor, 1986). *De novo* synthesis and /or direct molecular replacement by acylation-reacylation may be responsible for this replacement which ultimately influences rhodopsin function in visual cells.

Phosphatidylethanolamine and phosphatidylserine are located in the outer disc membrane monolayer (Miljanich et al., 1981). Docosahexaenoic acid is the major fatty acid in these phospholipids in retina and rod outer segments. In the present study the level of this fatty acid in the rod outer segment was 57% (w/w) and 63% (w/w) in phosphatidylethanolamine and phosphatidylserine respectively after feeding a diet containing high n-3 fatty acid. Docosahexaenoic acid is the principal component of dipolyunsaturated phospholipids found in bovine and frog rod outer segments (Aveldano, 1989; Aveldano & Bazan, 1983; Wiegand & Anderson, 1983). The relationship between fatty acid composition in phospholipid with asymmetric distribution in excitable membranes is not clearly established. However, the peculiar feature of membrane phospholipid distribution (Miljanich et al., 1981) with a high concentration of C22:6n-3 which can be changed by dietary fat provides insight into the possible role of phospholipid in membrane functions such as rhodopsin movement in photoreceptor cells.

Dietary Fat and Very Long Chain Fatty Acids

General compositional features of very long chain fatty acids in the rod outer segment was similar to that reported before (Suh et al., 1994; Aveldano & Sprecher, 1987). But, in the present experimental conditions, one more n-6 pentaenoic very long chain fatty acid was identified. Thus, it is now clear that very long chain fatty acids present as either n-6 tetraenes and pentaenes or n-3 pentaenes and hexaenes.

Increased n-3 fatty acid content in the diet significantly increased n-3 pentaenes of C24:5n-3, C26:5n-3, C30:5n-3, C32:5n-3 and C32:5n-3 type in phosphatidylcholine with a concomitant decrease in n-6 tetraenes of C24:4n-6, C26:4n-6, C28:4n-6, C32:4n-6 fatty acids and C34:4n-6 and n-6 pentaenes of C24:5n-6, C26:5n-6 and C32:5n-6 fatty acids in the rod outer segment in dark adapted animals. Similar changes occurred in fatty acids found in phosphatidylserine containing carbon chain length up to C26. However, phosphatidylcholine containing n-3 hexaenoic very long chain fatty acids did not respond to dietary treatment. This finding was also noticed with the previous diet regime containing 3.2% (w/w) C20:5n-3 and 1.2% (w/w) C22:6n-3 (Chapter IV). The dietary regime in the present study provided a higher dietary content of C22:6n-3 (3.4% w/w). It is puzzling that both studies exhibited the increase in C22:6n-3 in major phospholipids with no changes in hexaenoic very long chain fatty acid in phosphatidylcholine regardless of the level of

C22:6n-3 in diet. This unexpected result may suggest that C22:6n-3 is not a precursor for further elongation for hexaenoic very long chain fatty acids. It is known that very long chain fatty acids are synthesized by successive elongation from short chain precursors (Rotstein & Aveldano, 1988). Identification of these precursors and their further metabolism to very long chain fatty acids remains to be elucidated. Altered levels of very long chain fatty acids produced by diet treatment indicate that the *sn*-1 position of the glycerol backbone of phosphatidylcholine may be a main target for chain elongation. It is possible that specific chain elongase and activating enzymes may exist in the retina. Certainly, very long chain fatty acyl CoA synthetase has been found in liver peroxisomes (Singh et al., 1984). The implication of alteration of very long chain fatty acids by dietary treatment is still not known. It may be involved in rhodopsin movement since these very long chain fatty acids have a high affinity of bonding with rhodopsin (Aveladano, 1988) or are involved in membrane signal transduction which explored the effect of tetraenoic very long chain fatty acid on activation of protein kinase C in rat brain (Hardy et al., 1994) and Ca^{2+} modulation in human neutrophills (Hardy et al., 1995).

It has been reported that liver contains a retroconversion process to produce a C2 unit through one cycle of β-oxidation (Gronn et al., 1990 & 1991). This C2 unit may then be a carbon source for chain elongation. This retroconversion and chain elongation combination may play an important role in the synthesis of very long chain fatty acids. Thus, the findings of this experiment suggest that in the retinal inner segment and/or rod outer segment, this retroconversion system may occur for assembly of membrane components.

Light Exposure on Long and Very Long Chain Fatty Acids

In the present study, the effect of continuous light exposure on change in membrane composition was studied. Light exposure caused reduction in C22:6n-3 with increasing levels of C18:0 in phosphatidylcholine and phosphatidylserine after feeding the high n-3 fatty acid diet. Animals fed diets containing a low n-3 fatty acid content showed a decrease in level of C20:4n-6 in phosphatidylethanolamine and C22:6n-3 in phosphatidylserine. This finding agrees with the previous studies that retinal phospholipid fatty acid molecular species containing C22:6n-3 was reduced by light exposure (Wiegand et al., 1983 & 1995). Penn & Anderson (1997) and Penn & Thum (1987) suggests that the reduction of C22:6n-3 with bright light rearing may be just the adaptation these rats require for optimal retinal function in a new environment. This light induced reduction in long chain fatty acid is mediated by phospholipase A_2 (Jung & Reme, 1994; Reinboth et al., 1996; Birkle & Bazan, 1989) and may be involved in controlling biosynthesis of prostaglandins, leukotrienes, and other inflammatory mediators (Reinboth et al., 1995 & 1996).

Light treatment also reduced n-3 pentaenoic C24:5, C26:5 and C28:5n-3 and n-3 hexaenoic C26:6 and C28:6 fatty acids in phosphatidylcholine. Although not significantly different from dark adapted level, both total n-6 tetraenes and pentaenes and n-3 pentaenes and hexaenes were reduced by light exposure. This reduction occurred more in n-6 very long chain fatty acids than in n-3 very long chain fatty acid in animals of both diet treatments. This reduced level of n-6 very long chain fatty acid was not associated with C20:4n-6 since this fatty acid in phosphatidylcholine was not affected by light exposure. Thus, this data suggests that very long chain fatty acids undergo a different metabolic process compared to long chain fatty acids following light exposure. This finding also suggests that light also activates phospholipase A_1.

The present experiment demonstrated that free fatty acid and triglyceride were involved in the metabolism of the C20:4n-6 and C22:6n-3 lost from phospholipids after light exposure for 48 hours. Unlike the phospholipid response to light, the free fatty acid and triglyceride fractions contained increased levels of both C20:4n-6 and C22:6n-3 after light exposure in animals of both diet treatments. This suggests that free fatty acid and triglyceride are the reservoir for both C20:4n-6 and C22:6n-3 to enable recycling of essential fatty acid after light exposure.

One of the interesting finding of this experiment was that within the same n- series of fatty acids, different degrees of unsaturation respond differently to light treatment. For example, both the ratio of n-6 tetraenoic to pentaenoic very long chain fatty acid and the ratio of n-3 pentaenoic to hexaenoic very long chain fatty acid increased after light exposure. This implies that each n-6 and n-3 very long chain homologue has specific metabolic functions that behave differently in the photoreceptor membrane. Light exposure obviously activates more n-6 tetraenes and n-3 pentaenes than n-6 pentaenes and n-3 hexaenes. Feeding a diet high in n-3 fatty acids exhibited the highest ratio of n-3 pentaenoic to hexaenoic very long chain fatty acid of C26 to C32. These data imply that pentaenoic very long chain fatty acids respond more to diet and to light treatment.

Dietary Fat and Light on Rhodopsin Function

Rhodopsin content and rhodopsin phosphorylation were determined to identify the relationship between dietary fat and retinal functions. The present experiment demonstrated that rhodopsin phosphorylation *in vitro* was proportional to existing rhodopsin level. Rhodopsin content and its phosphorylation in the rod outer segment were higher in dark adapted animals after feeding a low n-3 fatty acid diet compared to animals fed a low n-3 fatty acid diet. Initially light exposed animals fed a low n-3 fatty acid lost more rhodopsin content of the dark adapted levels resulting in less rhodopsin phosphorylation compared to animals fed a high n-3 fatty acid diet. Therefore, the loss of rhodopsin function after light exposure was attenuated by feeding a high n-3 fatty acid diet. Whether this change has impact on physiological function is not known. Together with the fatty acid data, light evoked reduction of long and very long chain fatty acid may be associated with a reduced level of rhodopsin after light exposure.

In conclusion, it is clear that both long and very long chain fatty acids respond to dietary fat and light exposure. Very long chain fatty acid must be synthesized from shorter chain precursors present in the diet, but unknown mechanisms are involved in their metabolism. This experiment also reports for the first time light-induced loss of very long chain fatty acids. Light activates the synthesis of n-6 tetraenoic and n-3 pentaenoic very long chain fatty acids. All of these changes may be associated with retinal function, since rhodopsin content and rhodopsin phosphorylation was reduced by light exposure. Thus diet may be an important determinant of retinal fatty acid composition and function.

Chapter VI.

Dietary n-3 Fatty Acids Alter Rhodopsin Content and Rhodopsin Regeneration in Rat Retina Following Light Exposure.

INTRODUCTION

Diet induced fatty acid composition changes in neuronal tissues (Conner et al., 1990; Philbrick et al., 1987) affect the membrane protein functions (Hargreaves et al., 1987a & 1987b). This suggests that the microenvironment of the lipid bilayer plays an important role for the function of proteins. Many studies have focused on the physiological functional changes in the retina after feeding different diet regimes (Neuringer et al., 1986; Weisinger et al., 1996a & 1996b). However, the effect of diet on biochemical functions receive little attention. Rhodopsin is embedded tightly in the lipid bilayer of the disk membrane in retina which contains high levels of polyunsaturated fatty acids, especially docosahexaenoic acid (C22:6n-3). The retina also contains significant amounts of novel polyenoic very long chain fatty acids which comprise chain lengths of up to C36 (Aveldano 1988; Chapter IV & V). The relationship between these fatty acids and rhodopsin function has not been studied.

Rhodopsin regeneration is the process of light adaptation. The balance between photolizing and regeneration of rhodopsin is important to maintain 'photostasis' of visual cells. When rhodopsin resides in a delipidated environment, it is not able to regenerate rhodopsin, but regeneration is restored by addition of phospholipids (Shichi, 1971), especially phosphatidylethanolamine (Zorn & Futterman, 1971). Bush et al. (1994) have reported that rats fed safflower oil in the diet exhibit a slower rhodopsin regeneration rate than animals fed soybean oil. These results suggest that lipid, perhaps of the n-3 type, is important for regeneration of rhodopsin.

Thus, it is reasonable to predict that slight changes in membrane lipid in retina indicated by dietary fat may result in changes in rhodopsin function. In this regard, dietary fat with a high or a low n-3 fatty acid was fed to weaning rats and rhodopsin content and rhodopsin regeneration were measured *in vivo* and *in vitro*. Lipid peroxide level in the retina was also measured.

MATERIALS AND METHODS

Animals and Diets

Forty eight male weaning Sprague-Dawley rats were randomly assigned to two diet groups differing in the n-3 fatty acid composition containing either a high (4.8% w/w) n-3 fatty acids or a low (1.1% w/w) n-3 fatty acid level. Shark oil was used as the source of C22:6n-3. Animals were housed in a group of 3 under cyclic light with 12 hour dark and 12 hour light with the intensity at the front of the cage of 100 lux (1.54 Quantum, $\mu Em^2 sec^{-1}$) from cool, white fluorescent light. Water and food were fed ad libitum. The fat mixture and fatty acid composition of diets fed is presented (Table VI-1)

Table VI-1. Fatty acid composition of experimental diets.

Diet	High n-3	Low n-3
Fatty acid (% w/w)		
C14:0	2.4	1.9
C16:0	23.6	23.0
C18:0	47.4	50.4
C18:1n-9 + n-7	5.7	4.4
C18:2n-6	11.3	16.1
C18:3n-3	0.1	1.1
C20:4n-6	0.3	-
C20:5n-3	0.9	-
C22:6n-3	3.5	-
ΣSAT[a]	76.4	78.2
ΣMono[b]	7.0	4.5
Σn-6[c]	11.8	16.1
Σn-3[d]	4.8	1.1
P/S ratio[e]	0.22	0.22

Fatty acids with chain length greater than C24 were present less than 0.05% (w/w) in both diets. [a] ΣSAT, sum of saturated fatty acids; [b] ΣMono, sum of mono unsaturated fatty acids; [c] Σn-6, sum of n-6 fatty acids; [d] Σn-3, sum of n-3 fatty acids; [e] P/S ratio, polyunsaturated to saturated fatty acid ratio.

and reflects a typical North American dietary fat intake.

After 6 weeks of feeding, half of the rats in each diet group were transferred to polypropylene cages housed in pairs and were exposed to 48 hours of continuous light at the intensity of 1000 lux (12 Quantum, μEm^2sec^{-1}). The other half of each diet group were kept in complete darkness as a control. Diets were prepared weekly, kept in the freezer and fed to animals every other day. No lipid peroxides were observed in the diets fed (data not shown). Body weight was taken weekly and weight gain from weanling (55.4 ± 10.9 g) to 6 weeks of age (353.0 ± 35.0 g) was 6.2 g per day. No effect of dietary treatment was observed for either body weight or weight gain.

Isolation of Retina

Animals were sacrificed by decapitation. All isolations were performed at 4°C. Retinas were dissected either in dim red light for dark adapted animals or in ambient laboratory light for light exposed groups and kept at -70°C until analysis. For the analysis of rhodopsin content, light exposed animals were returned to darkness for 30 min before being sacrificed in dim red light.

Lipid Peroxides

Lipid peroxides in the retina were measured as thiobarbituric acid reactive substances (TBARS) based on the method described by Yagi (1987). This method was originally developed for human plasma and serum lipid peroxides and modified for retinal analysis. Retinas of either dark adapted or light adapted rats were homogenized in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1.5 mM BHT at 4°C. The homogenate was centrifuged at 12,000 g using a microcentrifuge at 4°C for 10 min. The resulting pellet was resuspended in 0.1 ml of the above solution. Eighty µl of retinal homogenate was mixed with 1 ml of 1/12 N H_2SO_4 and 0.12 ml of 10% phosphotungstic acid. Twenty µl was taken for protein analysis (Lowry et al., 1951). After standing for 5 min, the mixture was centrifuged at 3,000 g for 10 min. The supernatant was discarded and the pellet was again mixed with 2.0 ml of 0.083 N H_2SO_4 and 0.3 ml of 10% phosphotungstic acid and centrifuged at 3,000 g for 10 min. The resulting sediment was suspended with 0.55 ml of distilled water and mixed with the reaction mixture containing 0.3 ml 0.67% thiobarbituric acid, glacial acetic acid (1:1) and 0.15 ml of 5 mM BHT. The mixture was heated at 95°C for 1 hour. After cooling, 2.0 ml of n-butanol was added and the mixture was shaken thoroughly. After centrifugation at 3,000 g for 15 min, the n-butanol layer was measured using a luminecence spectrometer (LS 50B, Perkin Elmer, Buckinghumshire, England) at 553 nm with excitation at 515 nm. The data was expressed in terms of malondialdehyde equivalent (nmole/mg protein) using 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MO) as a standard.

Rhodopsin Measurement

Rhodopsin content of the retina was determined from animals of either dark adapted or light exposed groups. Rhodopsin was extracted (Fulton et al., 1982) with a slight modification using 1% (w/v) octylglucoside in 0.067 M phosphate buffer (pH 6.7). Each extract was scanned from 270 nm to 700 nm using a Hewlett Packard 8452A diode-array spectrophotometer in the presence of 0.1 M hydroxylamine in the complete darkness. And then it was scanned at 5, 10, 15, 30, 60, 120, 180, 240 and 480 seconds after bleaching with a cool white fluorescent light with the intensity of 1,000 lux (14.4 Quantum, μEm^2sec^{-1}) at a distance of 30 cm from the cuvette. Rhodopsin level was calculated by the change in absorption at 498 nm absorption before and after bleaching for 360 seconds. A molar extinction coefficient of 40,000 (Hubbard et al., 1991) was used for conversion of the measured optical density to concentration. The measurement at each time point was used to calculate the kinetics of rhodopsin bleaching.

Rhodopsin regeneration

For measurement of rhodopsin regeneration, animals were divided into 3 groups from each diet treatment. The first group was maintained in total darkness for 48 hours as a control. A second group of animals was exposed to light for 48 hours. The third group of animals was exposed to light for 48 hours and returned to the dark for 30 min. Retinas from both the first and the third groups were isolated in dim red light. The retinas in the second group were isolated in bright light to maintain the bleached level of visual pigment *in vivo*. Two retinas were pooled and homogenized in 1 ml of 0.067 M phosphate buffer (pH 6.7) for measurement of rhodopsin recovered *in vivo* and *in vitro*. A portion of the homogenate was also taken for protein analysis (Lowry et al., 1951).

In vivo

Half of the pooled retinal homogenate was used for measurement of rhodopsin content as indicated above. The homogenate was incubated in the dark with 3.5 μl ethanol for 1 hour prior to extraction of rhodopsin in 1% (w/v) octylglucoside in 0.067 M phosphate buffer (pH 6.7). Other steps were the same as described above. Rhodopsin of the light exposed group (second group) in each diet treatment was barely detectable by difference in spectra at 498 nm. Therefore, it was reasonable to measure rhodopsin regeneration from the third group since for this group rhodopsin was completely bleached and then regenerated when returned to the dark. Rhodopsin regeneration was calculated as the % of rhodopsin present in the third group to the rhodopsin present in dark adapted group (first group).

In vitro

The regeneration of the rhodopsin in retinal homogenates from the other half was determined by incubation with 11-*cis*-retinaldehyde (supplied by Dr. Crouch RK from Medical University of South Carolina, SC) in the dark. First, the retinal homogenates were bleached by exposure to light from two 150 W tungsten lamps (Zorn & Futterman, 1971, (1400 lux)) at a distance of 60 cm for 20 min at 22°C. In these bleached samples, 25 uM 11-*cis*-retinaldehyde in ethanol (final concentration, 0.7% v/v) was added in dim red light and incubated in the dark at room temperature for 1 hour. The extraction of rhodopsin was carried out as described before. Percent regeneration was determined by the rhodopsin content recovered from the second and the third group and was calculated based on the dark adapted level with no 11-*cis*-retinaldehyde added (*in vivo* dark adapted).

Statistical Analysis

The effect of light and diet on rhodopsin content and retinal lipid peroxide level was analyzed by two-way analysis of variance with light and diet as the main factors. The significant test of the main factors was based on a Duncan's multiple range test (Steel & Torrie, 1990). For analysis of rhodopsin regeneration, a two-way analysis of variance with a split plot procedure was used. The test of the main factors was based on the comparision by least square means. The analysis tool was SAS Statical Software (SAS Institute Inc., 1985). All data is expressed as mean ± standard error of the mean (S.E.M.).

RESULTS

Effect of Dietary Fat and Light on Retinal Lipid Peroxidation

The level of lipid peroxides expressed as malondialdehyde equivalent (nmol/mg protein) in whole retina was influenced by dietary fat ($p<0.005$) and light ($p<0.002$) treatment. Dark adapted animals fed a high n-3 fatty acid diet exhibited a lower retinal peroxide value (0.46 ± 0.09) than animals fed a low n-3 fatty acid diet (0.64 ± 0.03). The peroxides were significantly increased in animals fed a high n-3 fatty acid (0.66 ± 0.03) by the light exposure at the intensity of 1000 lux. Although not significant, the same pattern was observed in animals fed a low n-3 fatty acid diet (0.8 ± 0.05).

Effect of Dietary Fat and Light on Rhodopsin Content

Typical absorption spectra of retinal rhodopsin before and after bleaching was illustrated in Figure VI-1. The peaks indicated by solid arrows exhibit the effect of dietary treatment in dark adapted retinas. The difference between solid and dotted arrow from the same dietary group reflects the effect of light treatment *in vivo*. Rhodopsin content in the whole retina was not associated with dietary high or low n-3 fatty acid fed (1.46 ± 0.08

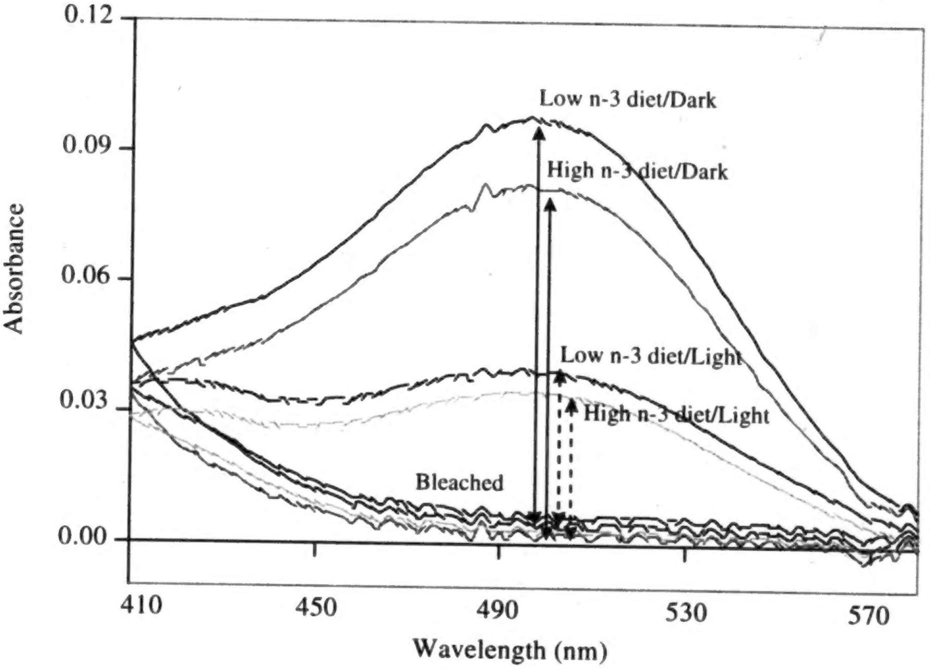

Figure VI-1. Typical absorption spectra from 410 nm to 570 nm of retinal rhodopsin following diet and light treatment. The difference between dark and light at 498 nm represents rhodopsin content of before and after bleaching for 480 seconds. The figure illustrates an example reflecting the diet treatment. The difference between solid and dotted arrows in the same diet verify the example of light treatment.

and 1.66 ± 0.08 nmole/mg protein respectively) (Figure VI-2). However, the level of rhodopsin in the ROS was significantly increased after feeding a low n-3 fatty acid diet (7.49 ± 0.49 nmol/mg protein) compared to a high n-3 fat diet (6.11 ± 0.18 nmole/mg protein). Visual pigments in both whole retina and ROS were significantly lost by light exposure for 48 hours *in vivo* compared to the level maintained in the dark (Figure VI-2). Animals fed a high n-3 fatty acid diet lost 61 % of rhodopsin in the retina and 51 % of rhodopsin in the ROS of dark adapted animals. The loss of rhodopsin after feeding a low n-3 fatty acid diet was slightly more than observed for the high n-3 fatty acid diet group, 67 % in retina and 59% in the ROS, respectively.

Effect of Dietary Fat and Light on Retinal Rhodopsin Kinetics

Rhodopsin disappearance after bleaching for each diet and light treated group is plotted in Figure VI-3. The kinetics of rhodopsin disappearances after bleaching was highest in animals fed a high n-3 fatty acid diet as a rate constant (*k*) 0.0023 versus 0.0013 for animals fed a low n-3 fatty acid diet. This implies that visual pigment in the high n-3 fatty acid environment quickly responds to light exposure. Once exposed to light *in vivo*, the rhodopsin disappearance was obviously slower than rhodopsin adapted in the dark.

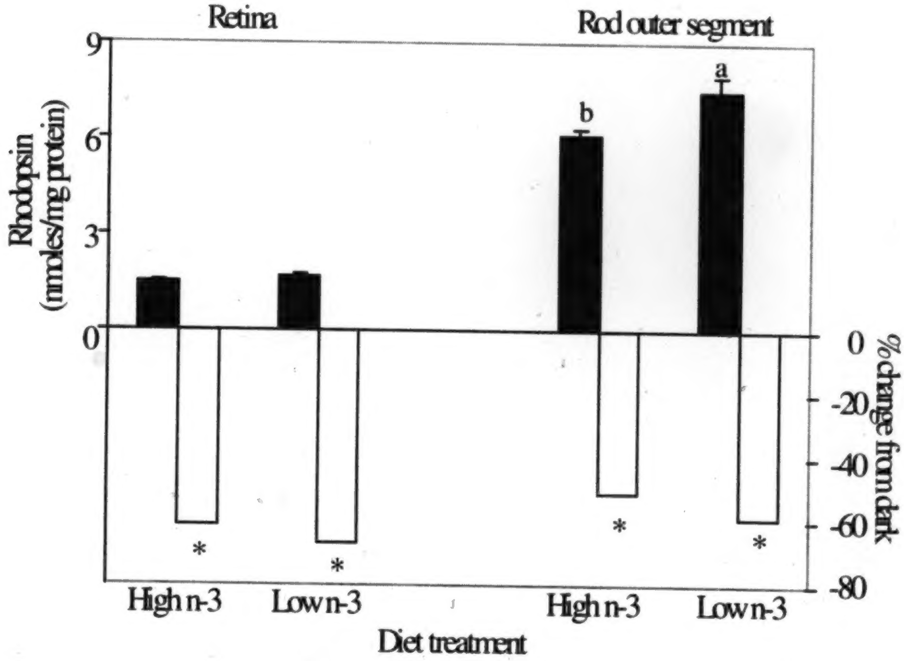

Figure VI-2. Effect of dietary fat and light on the level of rhodopsin in the retina and rod outer segment. The values are the mean (n=6) ± S.E.M. The level of rhodopsin in the retina was measured from one retina and for the rod outer segment from eight retinas. The value of % change from the level in the dark after light treatment was taken from the total mean of each group. In the rod outer segment, values without a common letter are significantly different, $p<0.05$. Significant effect of light was identified in both the retina and the rod outer segment (* $p<0.0001$). ■, dark; □, light.

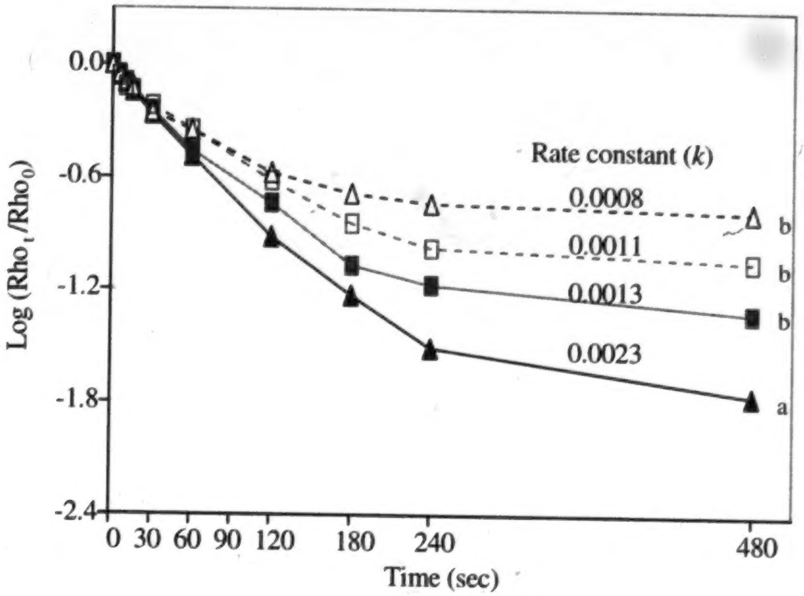

Figure VI-3. The effect of dietary fat and light on the kinetics of rhodopsin disappearance in rat retina. Rhodopsin was bleached for 5, 10, 15, 30, 60, 120, 180, 240 and 480 seconds. The Y-axis is the log of the rhodopsin bleached. The rate constants for rhodopsin disappearance are the mean (n=6) of each group. Values without a common letter are significantly different, p<0.05. Significant effect of light and interaction of light and diet were identified. —▲—, high n-3/dark; —■—, low n-3/dark; - △ - , high n-3/light; - □ - , low n-3/light.

Effect of Dietary Fat and Light on Retinal Rhodopsin Regeneration

The effect of dietary fat and light on the rhodopsin regeneration in whole retina was measured both *in vivo* and *in vitro* (Table VI-2; Figure VI-4). Animals initially dark adapted for 48 hours and fed a low n-3 fatty acid diet recovered the higher content of rhodopsin in both incubated with or without 11-*cis*-retinal for 1 hour in the dark (Table VI-2). However, once rhodopsin was bleached *in vivo*, the rhodopsin levels were not different between the two dietary treatments. Significant amount of rhodopsin was depleted in animals bleached *in vivo* for 48 hours and sacrificed in the light. The residual rhodopsin content was only 2.3 ± 0.2% and 1.7 ± 0.1% of the dark adapted level in animals fed a high n-3 fatty acid diet or the low n-3 fatty acid diet group, respectively.

Table VI-2. Recovery of rhodopsin *in vivo* and *in vitro* when incubated with 11-*cis*-retinal following diet and light treatment.

Diet	High n-3	Low n-3
(nmoles/mg protein)		
In vivo		
Dark adapted	1.22 ± 0.12^{b}	1.41 ± 0.04^{a}
Bleached	0.03 ± 0.01^{d}	0.02 ± 0.00^{d}
Bleached + Dark	0.32 ± 0.02^{c}	0.34 ± 0.02^{c}
In vitro (+ 11-*cis*-retinal)		
Dark adapted	0.93 ± 0.11^{b}	1.17 ± 0.09^{a}
Bleached	0.85 ± 0.15^{c}	0.90 ± 0.05^{b}
Bleached + Dark	0.92 ± 0.03^{b}	0.90 ± 0.05^{b}

The light treatment was described in the methods. Measurement of rhodopsin recovery *in vivo* was achieved by incubating retina in the dark with the addition of 3.5 μl ethanol for 1 hour. Measure of rhodopsin regeneration *in vitro* was achieved by bleaching retinas initially light treated for 20 min and then incubated in the dark with 25 μM 11-*cis*-retinal in 3.5 μl ethanol for 1 hour. The values are the mean (n=4) ± S.E.M. Values without a common letter *in vivo* or *in vitro* are significantly different, $p<0.05$. Significant effects of light ($p<0.0001$) and diet ($p<0.005$) were identified by two-way analysis of variance with a split plot procedure.

Regeneration of photolyzed rhodopsin *in vivo* was 27% in a high n-3 fatty acid group and 25% in a low n-3 fatty acid group (FigureVI-4). This implies that after 48 hours of light exposure, the recovery of rhodopsin in the first 30 min of dark was only 25% of its original levels. Rhodopsin initially bleached or bleached and then returned to the dark was regenerated *in vitro* with addition of 11-*cis*-retinaldehyde. In both light treatments, animals fed a high n-3 fatty acid diet had increased regeneration of rhodopsin. For example, in initially bleached animals, the regeneration was 69.9% in the high n-3 fatty acid group compared to 63.5% in animals fed a low n-3 fatty acid diet (Figure VI-4). In a group of animals initially bleached and then dark adapted for 30 min, feeding a high n-3 fatty acid diet increased the rhodopsin regeneration (75.6%) compared with feeding a low n-3 fatty acid diet (63.7%). This result implies that rhodopsin requires a specific environment for its regeneration.

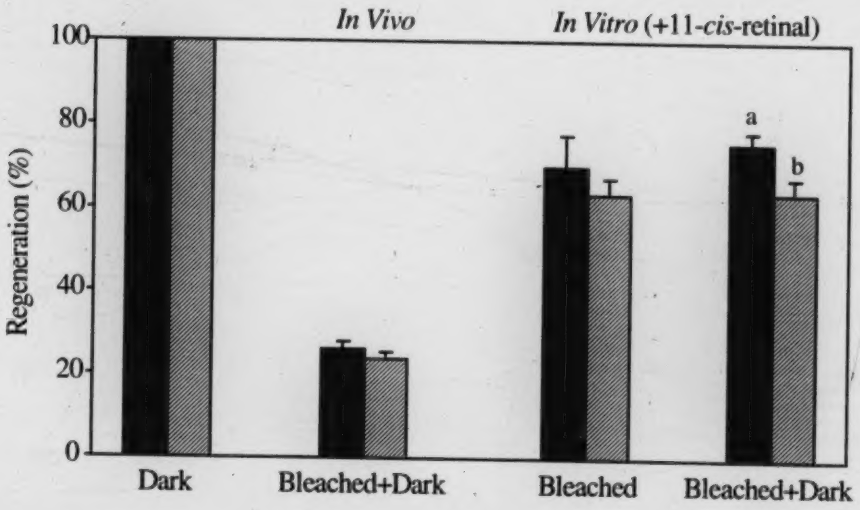

Figure VI-4. The effect of dietary fat and light on the regeneration of rhodopsin *in vivo* and *in vitro*. The values are mean (n=4) ± S.E.M. of % rhodopsin regenerated of dark adapted level with no 11-*cis*-retinaldehyde added. *In vivo*, retinas from rats exposed to light for 48 hours and then returned to the dark for 30 min were incubated in the dark with the addition of 3.5 µl ethanol for 1 hour. *In vitro*, retinas initially light treated *in vivo* were bleached for 20 min and then incubated in the dark with 25 µM 11-*cis*-retinal in 3.5 µl ethanol for 1 hour. Values without a common letter *in vitro* indicate significant effect of diet, $p<0.05$. ■, high n-3 diet; ▨, low n-3 diet.

DISCUSSION

Photoreceptor cells are designed to absorb light but they are also extremely susceptible to light-induced oxidation. C22:6n-3 is a likely target responsible for the susceptibility to oxidation (Wiegand et al., 1983). This fatty acid is easily oxidized and itself propagates radical chain reactions. Photoreceptor cells contain a variety of antioxidant enzymes and other compounds to protect against oxidation (Wiegand et al., 1986). Present experiment demonstrated that when animals were dark adapted for 48 hours, increasing membrane content of C22:6n-3 by feeding a high n-3 fatty acid diet actually showed lower retinal peroxide value compared to the low n-3 fatty acid diet. The peroxide levels were significantly increased after 48 hours of light exposure in animals fed a diet in a high n-3 fatty acid. This may cause light induced docosahexaenoic acid loss

(Wiegand et al., 1983 & 1986; Penn & Anderson, 1987). Studies have reported that this photooxidized oxidation can cause light induced photoreceptor cell damage (Kagan et al., 1973; Farnsworth & Dratz, 1976). The relationship between cellular lesions and the level of C22:6n-3 was not tested in this study. Reme et al. (1994) reported that feeding fish oil does not enhance susceptibility to acute rod outer segment disruption. They suggest that fish oil exerts a partial protective effect through formation of lipid mediators derived from eicosapentaenoic acid and arachidonic acid in retinal phospholipid.

Whole retinal and rod outer segment rhodopsin level in dark adapted animals was higher after feeding rats with a low n-3 fatty acid diet. However in both fractions, the loss after light exposure was greater in animals fed a low n-3 fatty acid diet. This suggests that a higher content of C22:6n-3 and increased pentaenoic very long chain fatty acids after feeding a high n-3 fatty acid diet (Chapter V) is able to protect against light induced rhodopsin loss. Some studies have suggested that a higher level of rhodopsin per retina or per rod outer segment length is associated with a greater susceptibility to light damage (Rapp et al., 1990; Penn et al., 1987).

The present study describes the effect of diet and light on rhodopsin regeneration in the whole retina. Consistent with other studies (Huang et al., 1990; Pepperberg et al., 1976; Jones et al., 1989), the present study found that rhodopsin was recovered *in vitro* by supplying of external 11-*cis* retinaldehyde, which is the chromophore in dark adapted retinas. Animals fed a high n-3 fatty acid diet regenerate more rhodopsin both *in vivo* and *in vitro.* This maybe associated with a higher content of C22:6n-3 and pentaenoic very long chain fatty acids (Chapter V). *In vivo* rhodopsin regeneration can be partly explained by increased rhodopsin disappearance kinetics after light exposure in animals fed a high n-3 fatty acid diet. This may result in a quick recycling of rhodopsin. Alternately, light induced increase in level of C22:6n-3 in the free fatty acid fraction may help to make the environment favourable for rhodopsin regeneration.

The present study supports the hypothesis that the microenvironment for the proper function of rhodopsin can be achieved and altered by dietary fat modulation. A high n-3 fatty acid diet was able to protect against light induced rhodopsin loss and increased rhodopsin regeneration *in vivo* and *in vitro*. Measurement of other parameters such as cGMP and rhodopsin kinase are needed to further enlighten the relationship between diet and rhodopsin regeneration related visual functions.

Chapter VII.

Dietary Polyenoic Fatty Acids Modulate the Profile of Long and Very Long Chain Fatty Acids (C24-C36), Rhodopsin Content and Kinetics in Developing Photoreceptor Cells

INTRODUCTION

Functions of the retina, measured by electroretinogram and visual evoked potentials, may mature earlier when infants are fed a nutritional regime containing long chain polyunsaturated fatty acids (Uauy et al., 1990; Birch et al., 1992 & 1993; Carlson et al., 1993). Deficiency of n-3 fatty acid is also known to be associated with loss of visual functions (Neuringer et al., 1984). It is generally believed that these functional changes in the retina are caused in some way by change occurring in the fatty acid constituents of phospholipids present in the retinal system associated with visual function. In this regard, increased dietary intake of n-3 fatty acids increases the n-3 fatty acid content of the rod outer segment (Suh et al., 1994; Lin et al., 1994). The location of compositional change occurring in the retina in response to dietary intake of fatty acid is not well known. Moreover the mechanisms linking change in the structural components of the retina to specific functional changes are also not known, but it is reasonable to speculate that mechanisms involve the photoreceptor cell and perhaps rhodopsin.

Development of the visual system follows a sequence of events, many of which involve synthesis of complex lipids to provide basic membrane structural components (Jumpsen & Clandinin, 1995). Retina membrane phospholipids, particularly phosphatidylethanolamine, contain high levels of C22:6n-3 (docosahexaenoic acid, DHA) (Birch et al., 1992). In the rod outer segment of the retina significant amounts of phosphatidylserine and phosphatidylcholine occur (Suh et al., 1994). In addition to DHA these two phospholipids contain very long chain polyenoic fatty acids from C24-C34 carbons in chain length of n-6 and n-3 type (Suh et al., 1994; Aveldano, 1987). The biological function and role of these very long chain components is not known.

Exposure to light stimulates turnover and recycling of membrane components in the rod outer segment of the retina (Birkle & Bazan, 1989; Wiegand et al., 1995). During development, the rod outer segment undergoes a transition in the composition of membrane phospholipids (Suh et al., 1996). It is also conceivable that these compositional changes may occur in concert with transitions in functions involving rhodopsin and the response by rhodopsin to light exposure.

Thus the present study was designed to examine the developmental change that occurs in the fatty acid composition of the rod outer segment after feeding diet fats that reflect the fatty acid composition of current infant formulas. To achieve this objective retinas were isolated from rats fed these diets for up to six weeks of life. The effect of diet treatment and developmental age on the fatty acid content of individual phospholipids, rhodopsin content and rhodopsin kinetics in response to exposure of the rod outer segment to light was also examined.

MATERIALS AND METHODS

Animals and Diets

Albino Sprague-Dawley rats were obtained for breeding from the University of Alberta vivarium. During breeding, three females and one male were housed together for a two week "mating" period. Females were then moved to individual cages. Room conditions were maintained at 21°C with 12 hour light and 12 hour dark cycle. Water and food were supplied ad libitum. Experimental diets were fed to dams following delivery of the rat pups. All litters were culled to twelve animals within 24 hours following parturition. Pups were sacrificed by decapitation in the dark at two, three and six weeks of age. Animals sacrificed at six weeks of age had been weaned at three weeks of age and were fed the same dietary regimen received by the dam.

Five semi-purified experimental diets containing 20% (w/w) fat and varying in fat composition were fed (Table VII-1; see appendix Table A-2). Diet fats were formulated to reflect the fat composition of a conventional infant formula providing a C18:2n-6 to

Table VII-1. Fatty acid composition of experimental diets.

Diet	Control[a]	AA[b]	DHA[b]	AA+DHA[b]	LA/LNA=4:1[c]
Fatty Acid (% w/w)					
C14:0	5.4	5.3	5.6	5.4	5.2
C16:0	13.3	13.4	13.2	13.2	13.0
C18:0	7.3	7.3	7.1	7.5	7.1
C18:1n-7+n-9	39.8	39.1	39.5	38.6	39.2
C18:2n-6	16.8	16.7	16.4	16.5	16.9
C18:3n-3	2.4	2.4	2.4	2.3	4.1
C20:4n-6	-	1.0	-	1.0	-
C22:6n-3	-	-	0.6	0.6	-
ΣSAT	38.4	38.5	38.3	38.4	37.4
ΣMono	42.1	41.1	41.8	40.7	41.4
Σn-6	16.8	17.8	16.5	17.7	16.9
Σn-3	2.6	2.6	3.4	3.1	4.1
C18:2n-6/C18:3n-3	7.0	7.1	6.9	7.1	4.1

[a] Fatty acid composition of the fat blend of an existing infant formula. [b] Control fat blend with addition of either AA, DHA, or AA and DHA mixture. [c] Control fat blend with increased level of LNA. Abbreviations used: AA, arachidonic acid; DHA, docosahexaenoic acid; LA, linoleic acid; LNA, linolenic acid; ΣSAT, total saturated fatty acids; ΣMono, total mono unsaturated fatty acids; Σn-6, total n-6 fatty acids; Σn-3, total n-3 fatty acids. Both C24:0 and C24:1 exist less than 0.18% (w/w) in each diet.

C18:3n-3 ratio of 7.0:1 (control fat blend; SMA®, Wyeth-Ayerst Laboratories, Radnor, PA). C22:6n-3 and C20:4n-6 were not present in this fat blend. Three experimental diets were prepared by addition of various triglycerides to provide AA and DHA in the basic control fat blend as follows: i) control fat blend was modified to contain 1% (w/w) AA; ii) control fat blend was modified to contain 0.6% (w/w) DHA; iii) control fat blend was modified to contain both 1% (w/w) AA and 0.6% (w/w) DHA (Table VII-1). Another diet was formulated to increase the level of C18:3n-3 to provide a C18:2n-6 to C18:3n-3 ratio of 4:1 (Table VII-1). The AA and DHA triglycerides utilized were obtained from single cell oils (Martek Corporation, Columbia, Maryland).

Isolation of Retina and Rod Outer Segment

All procedures were performed at 4°C under dim red light. Retinas were isolated (Fumiyuki et al., 1989) from the animals dark adapted overnight (12 hours). Twelve retinas were pooled for separation of the rod outer segment (ROS). The preparation of ROS by discontinuous sucrose gradient centrifugation followed the method developed by Stinson et al. (1991a). Aliquots of ROS were evaluated by microscopy and polyacrylamide gel electrophoresis (Laemmli, 1970). In the gel, opsin with a molecular weight of 35,000 daltons was present in ROS membranes.

Lipid Analysis

Lipids from ROS were extracted and partitioned according to the procedure of Folch et al. (1957). All organic solvent used contained 1 ppm ethoxyquin. Phospholipid classes were separated by spotting on hexane prewashed silica-gel TLC H-plates (20 x 20 cm) developed in chloroform:methanol:2-propanol:0.25% (w/v) KCl:triethylamine (30:9:25:6:18, by vol) (Touchstone et al., 1980) for 1.5 hours followed by chloroform: methanol:1-propanol:0.25% (w/v) KCl: triethylamine (30:9:25:6:18, by vol) for 1.5 hours. With these two systems the phosphatidylcholine fraction was further separated from sphingomyelin. TLC plates were visualized with 0.01% (w/v) anilino naphthalene sulfonic acid in water. Under the U.V. light only phosphatidylcholine contained two bands, upper and lower which were collected together. The upper band contained more very long chain fatty acids (data not shown). Each phospholipid was recovered and fatty acid methyl esters were prepared with 14% (w/w) boron trifluoride-methanol as described by Morrison and Smith (1964).

Analysis of Fatty Acid Methyl Esters

Fatty acid methyl esters were separated and on a polar BPX70 column (25 m x 0.22 mm I.D.) using a Hewlett Packard 5890 GLC equipped with a Vista DS 654 data system, as described earlier (Suh et al., 1994). Double bond numbers and n-series of very long chain fatty acids were confirmed by argentation TLC and GC-MS (Suh et al., 1994). Data were expressed as a percentage of the area count for an individual fatty acid relative to all fatty acids combined with no response factor correction.

Rhodopsin Measurement

Rhodopsin content and kinetics from retinas of each dietary group was determined in 2, 3 and 6 week old rats. Animals were dark adapted overnight for 12 to 14 hours prior to taking these measurements. Care was given to carry out all procedures in complete darkness or in dim red light. After isolation, retinas were immediately frozen in liquid nitrogen and stored at -70°C until analysis. Rhodopsin was extracted by the method of Fulton et al. (1982) with a slight modification using 1% (w/v) octylglucoside. Octylglucoside affords rapid solubilization of the disc membrane with non-denaturing properties and is highly soluble (Stubbs et al., 1976). Each retina was homogenized in 0.5 ml of 0.067 M phosphate buffer (pH 6.7) and 10 µl was taken for protein analysis (Lowry et al., 1951). The homogenate was transferred to an Eppendorf tube with an additional 1 ml of buffer and spun at 14,000 g for 30 min. Extraction was started by the addition of 0.45 ml of 1 % (w/v) n-octyl $_{\beta\text{-D}}$-glucopyranoside in 0.067 M phosphate buffer (pH 6.7) to the pellet with continuous shaking for 2 hours at 4°C. After extraction, 50 µl of 1 M hydroxylamine at pH 7.0 was added to each tube and then a clear rhodopsin extract was obtained by centrifugation at 14,000 g for 30 min. Each extract was scanned from 270 to 700 nm using a Hewlett Packard 8452A diode-array spectrophotometer in the complete darkness and then scanned at 5, 10, 15, 30, 60, 120, 180, 240, and 360 seconds during bleaching with a cool white fluorescent light (Kuhn & Wilden, 1982) with the intensity of 1,000 lux (14.4 Quantum, $\mu Em^2 sec^{-1}$) at a distance of 30 cm from the cuvette. Rhodopsin level was calculated by the change in absorption at 498 nm before and after bleaching for 360 seconds. A molar extinction coefficient of 40,000 (Hubbard et al., 1971) was used for conversion of the measured optical density to concentration. The measurement at each time point was used to calculate the kinetics of rhodopsin bleaching.

Statistical Analysis

The effect of diet and age on the fatty acid composition of phospholipid in the ROS and on the rhodopsin content of the retina was analyzed by two-way analysis of variance with diet and age as the main factors. The test of the main factors was based on the comparison by least square means. The analysis tool was SAS Statistical Software (SAS Institute Inc., 1985). All data is expressed as mean ± standard error of the mean (S.E.M.).

RESULTS

All pups grew normally during the experiment. However animals from the control or DHA diet groups tended to have higher body weight at two and three weeks of age (data not shown). By the six weeks of age, animals from all diet treatments had similar body weights except for animals fed a LA/LNA ratio of 4:1. These animals showed a lower body weight.

Effect of Dietary Fat on Fatty Acid Composition of ROS

Dietary fat significantly altered the fatty acid composition of the major phospholipids of dark adapted ROS of two, three and six week old rats. In all phospholipids, total saturated and monounsaturated fatty acid content reached the highest level at two weeks and then decreased by time of weaning (data not shown). By six weeks of age total saturated fatty acid content increased but total monounsaturated fatty acid decreased further (data not shown). The results illustrated focus on the level of C20:4n-6, C22:6n-3 and n-6 and n-3 very long chain fatty acids (Table VII-2 ; Table VII-3).

Phosphatidylcholine

Supplying small amounts of C20:4n-6 in the diet increased the level of C20:4n-6 (Table VII-2) at 3 and 6 week old age. At theses ages, inclusion of C22:6n-3 in the diet increased the level of C22:6n-3. The diet providing both C20:4n-6 and C22:6n-3 resulted in a greater level of both C20:4n-6 and C22:6n-3 at all ages. The diet containing a LA/LNA ratio of 4:1 increased the level of C20:4n-6 and C22:6n-3 only at the 3 week stage, but not significantly different from that of the control diet. During the period of the experiment, the accretion of C22:6n-3 increased 2.6 fold between 2 and 3 weeks of age and further increased by 6 weeks. The level of C20:4n-6 decreased after weaning.

Phosphatidylethanolamine

A greater level of C22:6n-3 in phosphatidylethanolamine was observed at 2 and 6 weeks of age after feeding a mixture of AA+DHA in the diet (Table VII-2). Providing DHA in the diet increased the content of C22:6n-3 at 3 and 6 weeks of age, while decreasing C20:4n-6 at 3 weeks of age compared to other diets. Animals fed the control or the AA diet contained the lowest level of C22:6n-3. Continuous decrease in C20:4n-6 content was observed from two to six weeks of age. The overall decrease in C20:4n-6 for animals in all diet groups from preweaning to weaning was 40.5%. During this period, the level of C22:6n-3 increased 1.6 fold.

Phosphatidylserine

A major fatty acid observed in phosphatidylserine was C22:6n-3, comprising over 50% to 60% (w/w) (Table VII-2). This level was the highest among all phospholipids in the ROS. Higher content of C22:6n-3 at 3 weeks of age was found when providing DHA in the diet compared to the control diet. The characteristic changes exhibited in C20:4n-6 and C22:6n-3 over the experimental period were similar to that occurring in phosphatidylethanolamine. Decrease in the level of C20:4n-6 from 2 weeks to 3 weeks was 47.7% whereas accretion of C22:6n-3 was 1.5 fold during this period.

Very long chain fatty acids

In the ROS, very long chain fatty acid of n-6 and n-3 homologues with a carbon chain length of up to C36 exist exclusively in phosphatidylcholine. Predominant among these fatty acids were n-6 tetraenoic and n-3 penta and hexaenoic fatty acid of C32 and C34 carbons in chain length. The level of n-6 pentaenoic very long chain fatty acid was minor. These observations characterize all ages examined. Levels of these very long chain

Table VII-2. Effect of dietary content of C20:4n-6 and C22:6n-3 on the level of C20:4n-6 and C22:6n-3 in phospholipids of rod outer segment of 2, 3 and 6 week old rats.

Fatty acid (% w/w)	Diet Age (wk)	Control	AA	DHA	AA+DHA	LA/LNA=4:1	Significant Effects (p<) Age	 Diet	 Age*Diet
Phosphatidylcholine									
C20:4n-6	2	6.7 ± 0.4^{b}	6.6 ± 0.2^{b}	5.7 ± 0.1^{c}	8.2 ± 0.3^{a}	7.2 ± 0.3^{b}	0.0001	0.0001	0.0001
	3	6.9 ± 0.2^{bc}	7.8 ± 0.4^{a}	6.6 ± 0.1^{c}	7.7 ± 0.3^{ab}	7.5 ± 0.1^{ab}			
	6	3.6 ± 0.1^{b}	5.0 ± 0.1^{a}	3.6 ± 0.1^{b}	4.4 ± 0.1^{ab}	3.8 ± 0.1^{b}			
C22:6n-3	2	11.2 ± 0.7	12.0 ± 1.2	12.0 ± 0.7	16.2 ± 1.0	11.9 ± 0.8	0.0001	0.0001	0.0015
	3	30.2 ± 1.4^{bc}	29.2 ± 1.4^{c}	35.8 ± 0.7^{a}	35.2 ± 1.0^{ab}	34.7 ± 0.5^{ab}			
	6	31.5 ± 1.6^{c}	36.1 ± 1.6^{bc}	43.6 ± 2.4^{a}	44.6 ± 2.0^{a}	37.1 ± 1.9^{b}			
Phosphatidylethanolamine									
C20:4n-6	2	10.1 ± 0.3	10.9 ± 0.4	10.0 ± 0.2	10.1 ± 0.4	10.6 ± 0.5	0.0001	0.0001	ns
	3	5.6 ± 0.1^{b}	7.0 ± 0.4^{a}	5.5 ± 0.1^{b}	6.3 ± 0.1^{ab}	6.4 ± 0.2^{ab}			
	6	3.2 ± 0.1	3.9 ± 0.1	2.9 ± 0.1	3.4 ± 0.3	3.4 ± 0.1			
C22:6n-3	2	32.2 ± 0.7^{b}	31.8 ± 1.2^{b}	33.1 ± 0.8^{ab}	36.1 ± 0.5^{a}	34.8 ± 1.2^{ab}	0.0001	0.0001	ns
	3	50.5 ± 1.2^{c}	51.5 ± 0.9^{bc}	56.7 ± 0.7^{a}	53.3 ± 0.6^{bc}	55.2 ± 1.2^{ab}			
	6	52.9 ± 0.7^{c}	54.8 ± 1.3^{bc}	57.7 ± 0.8^{ab}	59.3 ± 1.9^{a}	55.7 ± 1.0^{abc}			
Phosphatidylserine									
C20:4n-6	2	4.8 ± 0.2^{c}	6.7 ± 0.3^{a}	5.8 ± 0.3^{b}	5.0 ± 0.3^{c}	4.7 ± 0.4^{c}	0.0001	0.0001	0.0001
	3	2.7 ± 0.1	3.0 ± 0.1	2.6 ± 0.2	2.7 ± 0.2	2.9 ± 0.1			
	6	1.7 ± 0.1	2.1 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	2.1 ± 0.1			
C22:6n-3	2	38.1 ± 1.0	38.4 ± 2.6	39.2 ± 1.9	41.7 ± 1.9	38.7 ± 2.1	0.0001	0.0046	ns
	3	54.0 ± 1.6^{b}	56.1 ± 0.6^{ab}	61.6 ± 1.4^{a}	58.9 ± 2.2^{ab}	57.1 ± 1.5^{ab}			
	6	59.9 ± 0.7	59.3 ± 0.7	63.0 ± 0.9	62.1 ± 0.9	60.7 ± 0.9			

Values are group means (n=5 or 6) ± S.E.M. For each n 12 retinas were pooled. Significant effects were identified by two-way analysis of variance procedures for age and diet. Values within a row having a different superscripts indicate that diet group within each age is different by comparison of least squares means, $p<0.01$.

Table VII-3. Effect of dietary content of C20:4n-6 and C22:6n-3 on the level of C32 and C34 very long chain fatty acids in phosphatidylcholine of rod outer segment of 6 weeks old rats.

Diets Fatty acids (% w/w)	Control	AA	DHA	AA+DHA	LA/LNA=4:1	Effect of Diet (p<)
n-6 very long chain fatty acids						
C32:4	0.56 ± 0.06^{ab}	0.61 ± 0.07^{a}	0.52 ± 0.04^{b}	0.62 ± 0.05^{a}	0.51 ± 0.02^{b}	0.0034
C34:4	0.30 ± 0.03^{ab}	0.38 ± 0.04^{a}	0.30 ± 0.04^{b}	0.36 ± 0.03^{ab}	0.31 ± 0.01^{ab}	0.0062
C32:5	0.06 ± 0.01^{a}	0.07 ± 0.01^{a}	0.02 ± 0.00^{b}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	0.0001
C34:5	0.03 ± 0.00	0.04 ± 0.01	0.08 ± 0.03	0.02 ± 0.00	0.03 ± 0.01	ns
Σn-6VLCFA	1.39 ± 0.14^{b}	1.63 ± 0.12^{a}	1.33 ± 0.09^{b}	1.45 ± 0.11^{ab}	1.35 ± 0.07^{b}	0.0001
n-3 very long chain fatty acids						
C32:5	0.30 ± 0.02^{b}	0.20 ± 0.02^{c}	0.37 ± 0.02^{a}	0.28 ± 0.02^{b}	0.31 ± 0.02^{b}	0.0001
C34:5	0.50 ± 0.03^{bc}	0.41 ± 0.04^{c}	0.69 ± 0.05^{a}	0.58 ± 0.02^{ab}	0.62 ± 0.04^{ab}	0.0008
C32:6	1.67 ± 0.23^{b}	1.24 ± 0.12^{c}	2.20 ± 0.12^{a}	1.93 ± 0.08^{ab}	1.83 ± 0.14^{b}	0.0001
C34:6	1.46 ± 0.16^{b}	1.34 ± 0.12^{b}	2.14 ± 0.21^{a}	2.03 ± 0.08^{a}	1.97 ± 0.10^{a}	0.0008
Σn-3VLCFA	5.07 ± 0.54^{bc}	4.13 ± 0.33^{c}	6.63 ± 0.42^{a}	5.92 ± 0.22^{ab}	5.72 ± 0.36^{ab}	0.0001

Values are group means (n=6) ± S.E.M. For each n 12 retinas were pooled. P values are obtained from two-way analysis of variance procedures for age and diet. Significant effects of age were identified for the above fatty acids except C34:5n-6 ($p<0.005$). Significant interactions were found in all n-3 very long chain fatty acids in above. Values within a row having a different superscripts are different by comparison of least squares means at the level of $p<0.01$ except C32:4n-6, C34:4n-6 and Σn-6VLCFA at $p<0.05$. AA, arachidonic acid; DHA, docosahexaenoic acid; Σn-6VLCFA, total n-6 very long chain fatty acids; Σn-3VLCFA, total n-3 very long chain fatty acids. Values of 2 and 3 weeks are not listed in this table.

fatty acids were significantly affected by the dietary fatty acid supplied (Table VII-3). Providing rats with AA or AA+DHA in the diet resulted in increased levels of C32:4n-6 and C34:4n-6 compared to the DHA diet at 6 weeks of age. The level of C32:5n-6 was higher by feeding the AA diet. This pattern was also recognized at 2 and 3 weeks of age (data not shown). Conversely, inclusion of DHA or AA+DHA in the diet increased the n-3 penta and hexaenoic fatty acid of C32 and C34 carbons in chain length. Animals fed the control or the AA diet exhibited the lowest level of n-3 very long chain fatty acids by 6 weeks of age. Feeding a LA/LNA ratio of 4:1 in the diet failed to exhibit the same level of n-6 tetraenoic and pentaenoic very long chain fatty acids compared to feeding the AA diet. Feeding a diet providing LA/LNA ratio of 4:1 also did not result in a higher level of C32:5n-3 and C32:6n-3 compared to feeding the DHA diet.

Changes in Long and Very Long Chain Fatty Acid Content of the ROS with Developmental Age

Accretion of both total n-6 tetraenoic and n-3 pentaenoic and hexaenoic very long chain fatty acids in phosphatidylcholine more than doubled between 2 and 3 weeks of age and then increased further by 6 weeks (Figure VII-1). During these periods, feeding AA in the diet further increased the level of total n-6 tetraenoic very long chain fatty acid compared to feeding DHA or feeding the diet providing a LA/LNA ratio of 4:1. Providing rats with DHA in the diet increased total n-3 penta and hexaenoic very long chain fatty acid at all ages. Unlike with n-6 tetraenoic very long chain fatty acids, total n-6 pentaenoic very long chain fatty acids decreased from weaning to 6 weeks of age.

Developmental changes in the ratio of C22:6n-3 to C20:4n-6 and n-3/n-6 very long chain fatty acid were also examined in the phospholipids of the ROS. The ratio of C22:6n-3 to C20:4n-6 continuously increased during development in phosphatidylcholine and phosphatidylethanolamine (Figure VII-2). The same pattern was identified in phosphatidylserine (data not shown). The highest ratio of C22:6n-3 to C20:4n-6 was found in rats fed the diet containing DHA. The ratio of n-3 very long chain fatty acids to

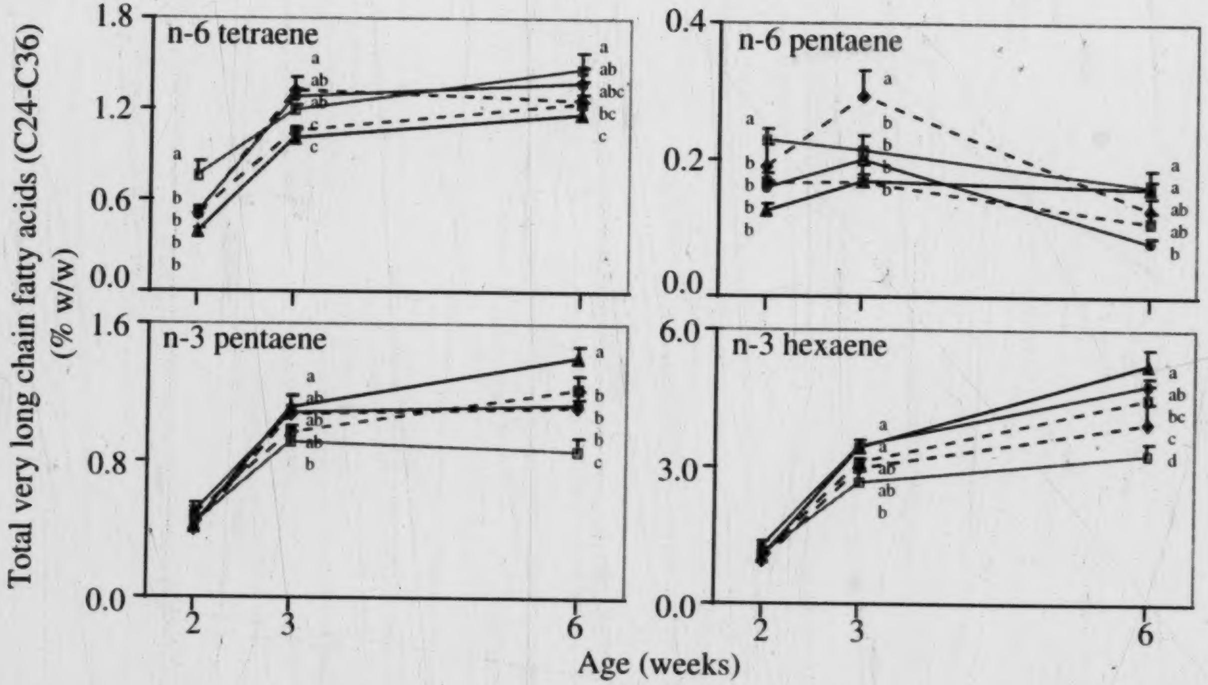

Figure VII-1. Very long chain fatty acid (C24-C36) content in phosphatidylcholine of rod outer segment of rat retina after supplying C20:4n-6 and C22:6n-3 in diet at 2, 3 and 6 weeks of age. Values given are means (n=6) ± S.E.M. For each n 12 retinas were pooled. Values without a common letter within each age are significantly different, $p<0.05$. Significant effects were identified by two-way analysis of variance procedure for age ($p<0.0001$) and diet ($p<0.0005$) in each very long chain fatty acid homologues. -◆- control; -□- AA; -▲- DHA; -◇- AA+DHA; -■- LA/LNA=4:1.

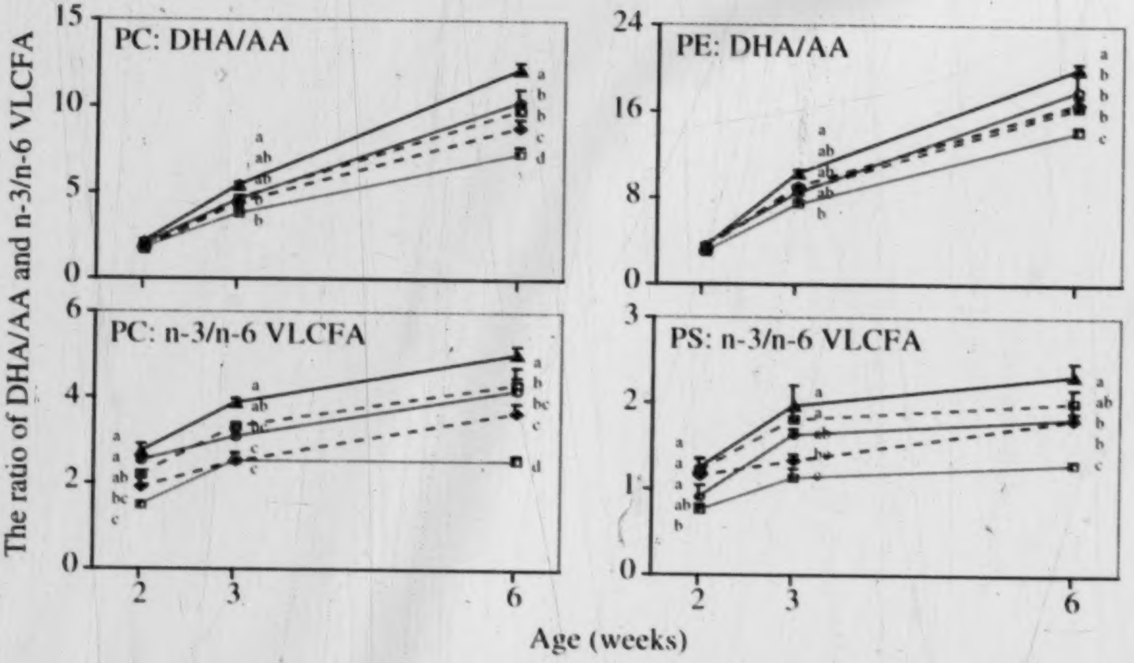

Figure VII-2. The ratio of C22:6n-3 to C20:4n-6 and n-3 to n-6 very long chain fatty acids in phospholipids of rod outer segment of rat retina after supplying C20:4n-6 and C22:6n-3 in diet at 2, 3 and 6 weeks of age. Values given are means (n=5 or 6) ± S.E.M. For each n 12 retinas were pooled. Values without a common letter within each age are significantly different, $p<0.05$. Significant effects were identified by two-way analysis of variance procedure for age ($p<0.0001$) and diet ($p<0.0001$) in each ratio. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DHA/AA, the ratio of docosahexaenoic acid to arachidonic acid; PC:n-3/n-6 VLCFA, the ratio of total n-3 to total n-6 very long chain fatty acids (C24-C36); PS:n-3/n-6 VLCFA, the ratio of total n-3 to total n-6 very long chain fatty acids (C24-C26). -◆- control; -□- AA; -▲- DHA; -○- AA+DHA; -■- LA/LNA=4:1.

n-6 very long chain fatty acids also increased with age in phosphatidylcholine and phosphatidylserine. In phosphatidylserine, the very long chain fatty acids were of carbon chain length up to C26. Inclusion of a small amount of DHA in the diet resulted in the highest ratio of n-3 very long chain fatty acids to n-6 very long chain fatty acids in phosphatidylcholine and phosphatidylserine, while the lowest ratio was observed in rats fed the diet containing AA.

Effect of Dietary Fat on the Rhodopsin Content of the ROS

To examine the relationship between dietary fat and retinal function, rhodopsin content was evaluated in the retina of animals at 2, 3 and 6 weeks of age. The rhodopsin content in the retina increased greatly with age when expressed as nmole/mg protein. Figure VII-3 illustrates the absorption spectrum of the pigment, which reflects the level of rhodopsin *in situ*. For example, retina from rats fed a diet containing AA+DHA was 1.6 times higher in rhodopsin content at weaning age (0.70 ± 0.08 nmole/mg protein) than prior to weaning (0.45 ± 0.01 nmole/mg protein). Rhodopsin content continuously increased to 1.02 ± 0.03 nmole/mg protein by 6 weeks of age. The highest rhodopsin content occurred in the retina of animals fed diets containing a small amount of AA and/or DHA at 6 weeks of age. When rhodopsin content in the animals fed the control diet is considered as 1, the highest relative content of rhodopsin was exhibited by animals fed the mixture diet of AA+DHA at 2, 3 and 6 weeks of age (1.24, 1.13 and 1.32, respectively). Providing animals with DHA in the diet also produced higher visual pigment content at 2 and 6 weeks of age (1.17 and 1.31, respectively). Animals fed the diet providing a LA/LNA ratio of 4:1 exhibited the lowest relative rhodopsin content at 2 and weeks of age (0.99 and 1.23 respectively).

Effect of Dietary Fat on Rhodopsin Kinetics

The effect of dietary fat treatment on the kinetics of rhodopsin disappearance at 6 weeks of age was significant ($p<0.05$). Rhodopsin disappearance after bleaching is

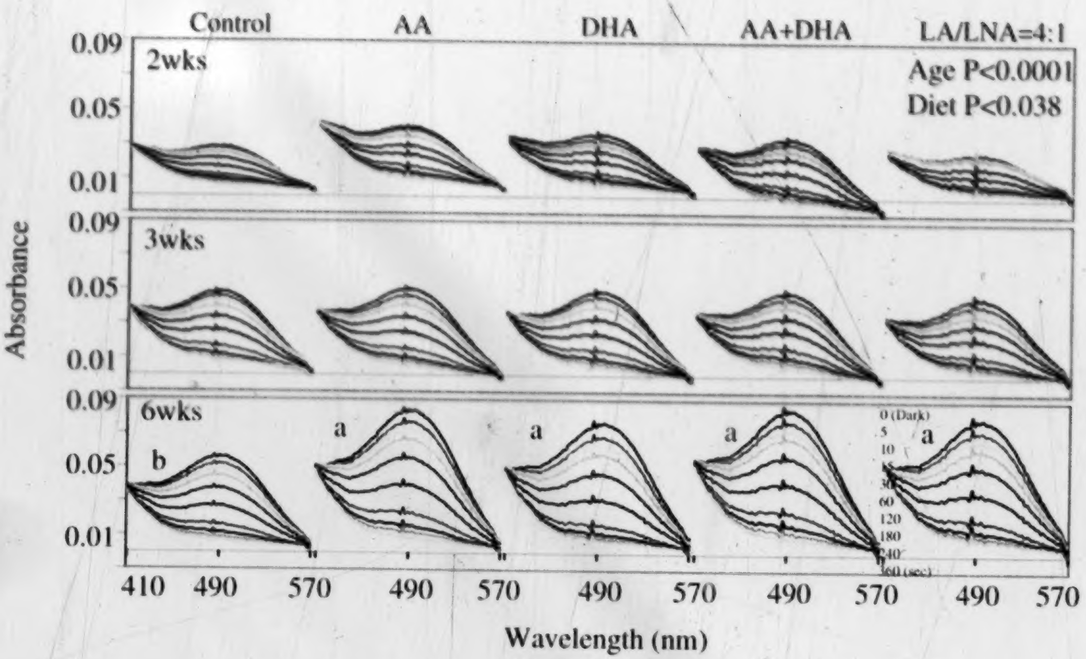

Figure VII-3. The absorption spectrum from 410 nm to 570 nm for retinal rhodopsin from rats fed experimental diets for 2, 3 and 6 weeks. Each spectrum represents means (n=5 or 6). Significant effects were identified by two-way analysis of variances procedure for age and diet. Spectra without a common letter in 6 weeks are different by comparison of least square means, $p<0.05$. The level of rhodopsin was calculated by the change in absorption at 498 nm before and after bleaching for 360 seconds. The rhodopsin levels (nmole/mg protein, mean ± S.E.M.) for 2, 3 and 6weeks old animals fed the control, AA, DHA, AA+DHA or LA/LNA=4:1 diet, respectively, are as follows: 2wks, 0.36 ± 0.04, 0.42 ± 0.03, 0.42 ± 0.01, 0.45 ± 0.01, 0.36 ± 0.04; 3wks, 0.62 ± 0.07, 0.62 ± 0.09, 0.65 ± 0.06, 0.70 ± 0.08, 0.72 ± 0.05; 6wks, 0.77 ± 0.07, 1.00 ± 0.06, 1.02 ± 0.03, 1.02 ± 0.05, 0.95 ± 0.07. The measurement at each time point, 0, 5, 10, 15, 30, 60, 120, 180, 240 and 360 seconds, was used to calculate the kinetics of rhodopsin bleaching illustrated in Figure VII-4.

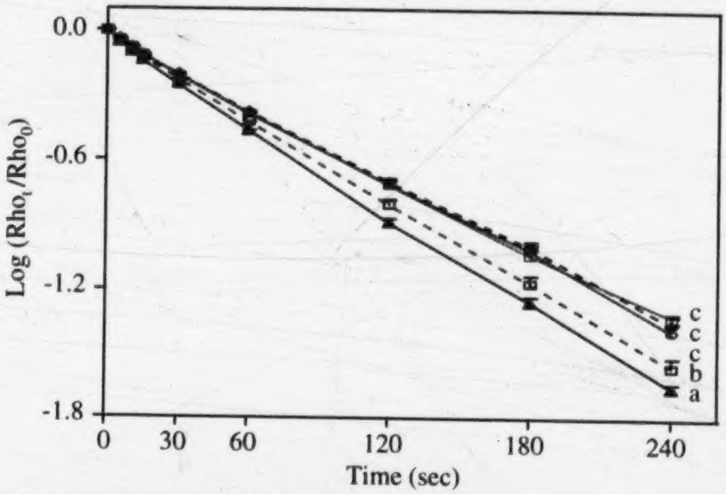

Figure VII-4. Effect of dietary content of C20:4n-6 and C22:6n-3 on the kinetics of rhodopsin disappearance in rat retina at 6 weeks of age. Rhodopsin was bleached for 5, 10, 15, 30, 60, 120, 180, 240 and 360 seconds. The Y-axis is the log of the rhodopsin bleached. Significant effects of diet were tested by least squares means comparison, $p<0.05$. The time (seconds) of half life of rhodopsin is also significantly different at $p<0.05$. The half life of rhodopsin and the rate constants (*k*) of rhodopsin disappearance for diet treatments, control, AA, DHA, AA+DHA and LA:LNA=4:1, respectively, are as follows (mean ± S.E.M., n=5 or 6): half life (seconds), 48.7 ± 2.3^{a}, 47.2 ± 0.7^{a}, 38.5 ± 1.4^{b}, 47.9 ± 1.5^{a}, 43.2 ± 1.4^{ab} ; rate constants (*k*, mean), 0.0024, 0.0024, 0.0030, 0.0025, 0.0028. Rho_t, dark adapted rhodopsin; Rho_0, bleached rhodopsin at time t. -◆- control; —□— AA; —▲— DHA; —θ— AA+DHA; -▣- LA/LNA=4:1.

illustrated for each diet group (Figure VII-4). The rate constant (*k*) of rhodopsin disappearance was represented by the slope determined from each bleaching time point (Figure VII-4). The kinetics of rhodopsin photolyzing after light exposure was the greatest in animals fed a small quantity of DHA (*k*, 0.0030), followed by animals fed the diet providing a LA/LNA ratio of 4:1 (*k*, 0.0028). During bleaching for 360 seconds, rhodopsin from animals fed the DHA diet disappeared in a short time compared to the rate of disappearance in animals fed the other diets (half life, 38.5 ± 1.4 seconds).

DISCUSSION

Relationship between Dietary Fat and Long Chain Fatty Acids Composition

Recently, the correlation between docosahexaenoic acid and visual function has been well documented (Neuringer et al., 1986; Pawlosky et al., 1997). However, these studies in animal models used extremely unbalanced n-6 to n-3 fatty acid diet. The present study demonstrates that small manipulations of dietary level of C20:4n-6 and/or C22:6n-3, within the physiological intake range (Clandinin et al., 1992), are important determinants of visual cell membrane fatty acid composition during development. The alteration of membrane fatty acid composition by diet may have significant implications for early retinal development related with rhodopsin function and visual cell renewal. The choice of infant formula or breast milk for full term and pre term infants or animals is associated with visual cell maturation and visual functions (Uauy et al., 1990 & 1992; Werkman & Carlson, 1996; Faldella et al., 1996). Dietary fatty acid related functional loss during development cannot be restored in later life even though that deficient fat component is restored (Neuringer et al., 1989). Therefore early dietary intervention is necessary, although optimum level of intake is not fully understood for different developmental stages.

In the present study, supplying a diet containing the C20:4n-6 and C22:6n-3 was intended to increase levels of both C20:4n-6 and C22:6n-3 in phospholipid of growing visual cells. Feeding this diet increased the content of C20:4n-6 and C22:6n-3 in phosphatidylcholine from weaning to post weaning ages. The level of both C20:4n-6 and C22:6n-3 is comparable to animals fed a diet containing DHA or AA alone. Thus, addition of C20:4n-6 to a diet containing C22:6n-3 did not compete with each other for acylation into the membrane phospholipid. Probably, synthesis of dipolyunsaturated phospholipid species is required by the rod outer segment (Aveldano & Bazan, 1983; Aveldano, 1989).

In the present study, a physiological intake level of DHA increased docosahexaenoic acid in phosphatidylcholine which caused a concomitant reduction in the level of arachidonic acid. However, this diet did not reduce the level of C20:4n-6 in phosphatidylethanolamine and phosphatidylserine, although the level of C22:6n-3 increased. This finding is similar to that observed in phosphatidylethanolamine from brain using similar dietary regimes (Jumpsen et al., 1997).

Retina possesses the enzymatic system to elongate and desaturate C18:3n-3 to C22:6n-3 (Wetzel et al., 1991). When rats were fed an increased level of C18:3n-3 without longer chain fatty acid in the diet, the animals exhibited a comparable amount of C22:6n-3 in phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine at weaning to those fed the DHA or fed the AA+DHA diet mixture. After weaning, providing an increased level of C18:3n-3 in the diet was inadequate in raising the level of C20:4n-6 and C22:6n-3 in phosphatidylcholine compared to animals fed AA or DHA in the diet. It appears that feeding preformed DHA or AA better supports increase in C20:4n-6 and C22:6n-3 levels in growing retina. It has been reported that C18:3n-3 is a less efficient precursor for docosahexaenoic acid synthesis (Anderson et al., 1990; Kohn et al., 1994) and docosahexaenoic acid is the preferred fatty acid for raising the level of C22:6n-3 in the retina (Anderson et al., 1990).

It is important to know the developmental profile of fatty acid during the period of rapid visual cell growth. These profiles can be an index for intervention by dietary treatment when the natural homeostasis is altered. From 2 weeks to 6 weeks of the experimental period, there was accretion of C22:6n-3 whereas C20:4n-6 decreased as a function of age. The changes were more distinct between preweaning and weaning. During this period C22:6n-3 increased 2.6 , 1.6 and 1.5 fold in phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, respectively while C20:4n-6 level at weaning decreased by 40% and 48% from 2 weeks of age in phosphatidylethanolamine and phosphatidylserine, respectively. This data agree with previous studies of human and animal retina (Bazan 1988; Martinez et al., 1988). Similar changes occur in the brain and liver during maturation (Martinez, 1988; Jumpsen et al., 1997). However, accretion of C20:4n-6 in phosphatidylcholine shows a different pattern during this period. The level of C20:4n-6 increased slightly after weaning and then fell significantly by 6 week of age. This suggests that each phospholipid metabolizes fatty acid differently. Together with other studies, this experiment provides evidence that the photoreceptor membrane fatty acid composition shifts from saturated to unsaturated and more specifically docosahexaenoic acid. This shift during maturation of visual cells is likely related to the physiological functional development of the retina.

Martinez (1988) suggests that the ratio of C22:6n-3/C20:4n-6 can be used as a marker of developmental changes in polyunsaturated fatty acid levels in brain and liver. In the present study, the ratio of C22:6n-3 to C20:4n-6 increased continuously during development in major phospholipids of the retina. For example, after feeding the DHA diet, the ratio of C22:6n-3 to C20:4n-6 in phosphatidylcholine was 2.12, 5.39, and 12.19 at 2, 3, and 6 weeks of age. A much higher ratio was found in phosphatidylethanolamine and phosphatidylserine as the content of C22:6n-3 in these phospholipids is high. The highest ratio was found in animals fed the DHA diet and the lowest ratio in animals fed the AA diet.

Relationship between Dietary Fat and Very Long Chain Fatty Acid Composition

This experiment provided evidence for the dietary response of very long chain fatty acids during developing stages of the photoreceptor. The level of total n-6 and n-3 very long chain fatty acids, respectively was 0.68% and 1.73% at 2 weeks, 1.48% and 4.54% at 3 weeks and 1.54% and 5.8% at 6 weeks from animals fed the AA +DHA diet. The level of total n-6 and n-3 fatty acids at 9 week old rat is 0.87% (not included n-6 pentaenes) and 5.87%, respectively (Suh et al., 1994). These results suggest that both n-6 and n-3 very long chain fatty acids are continuously increases until 9 weeks of age. This increase in the level of very long chain polyenoic fatty acid may be associated with visual cell development.

Inclusion of small amounts of the AA+DHA in the diet resulted in increased levels of C32:4n-6, C34:4n-6 and C34:5n-3, C32:6n-3, C34:6n-3 at 6 weeks of age. These fatty acid levels were comparable to those animals fed AA or DHA for n-6 and n-3 fatty acid, respectively. Supplying DHA in the diet significantly increased n-3 pentaenoic and hexaenoic very long chain fatty acids with significant reduction occurring in the n-6 tetraenoic and pentaenoic very long chain fatty acid level. Feeding a LA/LNA ratio of 4:1 in the diet failed to exhibit the same level of n-6 tetraenoic and pentaenoic very long chain fatty acids compared to feeding the AA diet. Feeding a LA/LNA ratio of 4:1 in the diet did not increase the level of C32:5n-3 and C32:6n-3 compared to feeding the DHA diet. Apparently feeding a LA/LNA ratio of 4:1 in the diet does not strongly support the level of C22:6n-3 and very long chain n-3 fatty acids present in growing visual cells.

Accretion of both total n-6 tetraenoic and n-3 pentaenoic and hexaenoic very long chain fatty in phosphatidylcholine more than doubled between 2 and 3 weeks of age and then increased further by 6 weeks. This increase may be related to the increase of C22:6n-3. Unlike with n-6 tetraenoic very long chain fatty acids, total n-6 pentaenoic very long chain fatty acids decreased from weaning to 6 weeks of age. This data is similar to that previously reported in that n-6 pentaenoic fatty acids predominate in neonatal rat and human fetal brain of 24 week of gestation, and that no n-6 tetraenoic acid are present beyond the neonatal period (Robinson et al., 1990b; Sharp et al., 1991). Since monoenoic very long chain fatty acid increases in parallel with the process of myelination (Martinez, 1991), it is reasonable to speculate that these very long chain fatty acids may be related to rhodopsin development.

The ratio of n-3 to n-6 very long chain fatty acids continuously increased from 2 week to 6 weeks of age. For example, the ratio of n-3 to n-6 very long chain fatty acid in phosphatidylcholine was 2.74, 3.85, and 4.99 at 2, 3, and 6 weeks of age after feeding the DHA diet. The highest ratio of n-3 to n-6 very long chain fatty acid occurred in animals fed the DHA diet. The lowest ratio of n-3 to n-6 very long chain fatty acid occurred in animals fed the AA diet. Together with the increased ratio of C22:6n-3 to C20:4n-6, this data suggests a developing photoreceptor cell fatty acid transition towards higher n-3 fatty acid. The importance of n-3 fatty acids in retinal function has been documented in animals (Neuringer et al., 1986; Weisinger et al., 1996a & 1996b) and humans (Uauy et al., 1990 & 1992; Carlson et al., 1993 & 1996).

Relationship between Dietary Fat and Rhodopsin

To evaluate the relationship between dietary fat and retinal function, rhodopsin content and rhodopsin disappearance kinetics were evaluated after light exposure. For example, retinas from rats fed a diet containing AA+DHA increased 1.6 times from preweaning to weaning and increased a further 1.5 times to 6 weeks of age. The 6 weeks of age value for rhodopsin lies within the range of whole eye rhodopsin levels reported in the literature for similar body weights (Huang et al., 1990). In the previous experiment, the level of rhodopsin at 9 weeks of age was between 1.5 and 1.7 nmoles/mg protein (Chapter VI). These data suggest that the retinas used in this experiment are still in developing stages. Thus, developmental increase in rhodopsin is associated with increasing levels of docosahexaenoic acid and very long chain fatty acids. Compared to the control diet, the highest relative content of rhodopsin is exhibited by animals fed AA+DHA at 2, 3, and 6 weeks of age. Considering that this diet increases both n-6 and n-3 long chain and very long chain fatty acids, it is reasonable to associate these changes in fatty acid composition with increased rhodopsin level. However, rhodopsin content also increased by 6 weeks of age in animals fed diets containing a small amount of AA, DHA or the diet providing a ratio of LA/LNA 4 to 1. For animals fed safflower oil (Bush et al., 1994) and fish oil (Reme et al., 1994) a significantly higher whole rhodopsin level is observed when compared to animals fed soybean oil (Bush et al., 1994). It is difficult to rationalize the increase in rhodopsin observed in animals fed safflower oil or fish oil compared to animals fed soybean oil, since safflower oil feeding results in deficiency of n-3 fatty acids and fish oil provides high n-3 fatty acids.

When initially dark adapted retinas *in vivo* were exposed to light *in vitro*, the rhodopsin disappearance kinetics in the retinas was quite different depending on the animals diet. Rhodopsin disappeared in a shorter time after light exposure in animals fed a small amount of DHA, followed by in animals fed a diet providing a LA/LNA ratio of 4:1. A similar finding is reported in Chapter VI. This suggests that n-3 fatty acid containing membrane components influence the rhodopsin response to light. Rhodopsin disappearance in a short time may result in more or quick rhodopsin regeneration (Chapter VI).

It was concluded that even small dietary amounts of C20:4n-6 and C22:6n-3 were sufficient to influence membrane long and very long chain fatty acid composition of the visual cell during development. Addition of AA or DHA individually in the diet caused imbalance in the level of long chain, very long chain fatty acids resulting in significantly high or low ratios of DHA/AA and n-3/n-6 very long chain fatty acids. Thus, the mixture of AA and DHA in the diet better supports a balance between n-3/n-6 fatty acids that seems to be needed to develop the highest relative rhodopsin content. Although functional roles for very long chain n-6 and n-3 fatty acids in photoreceptors still remain unclear, relationships exist between rhodopsin, docosahexaenoic acid and other very long chain fatty acids.

Chapter VIII.

C20:5n-3 is a Preferred Substrate for Synthesis of n-3 Very Long Chain Fatty Acids (C24-C36) in Retina.

INTRODUCTION

Fatty acid metabolism of long chain fatty acid *in vivo* and *in vitro* has been extensively studied in retina using several animals models. These studies show that the enzymes for elongation-desaturation, acylation-deacylation of C18:3n-3 or C20:5n-3 to C22:6n-3 appear to be present in the retina (Wetzel et al., 1991; Bazan et al., 1982a; Giusto et al., 1986). Studies using radiolabeled C22:6n-3 find that C22:6n-3 is selectively taken up by photoreceptors and then esterified into photoreceptors (De Tulco et al., 1990 & 1991; Wetzel et al., 1991). However, the metabolism of fatty acids of longer than carbon chain length 22 has not been studied even though a high concentration of very long chain fatty acids (C24-C36) occurs in photoreceptors (Suh et al., 1994; Aveldano, 1987 & 1988). Only a few studies have reported that these fatty acids are synthesized by successive elongation from shorter chain precursors in retina using ^{14}C-acetate *in vitro* (Rotstein & Aveldano, 1988; Rotstein et al., 1996). The synthesis from precursor fatty acids is not known when long chain fatty acids are given as the precursor. Since C18:3n-3, C20:5n-3 and C22:6n-3 can be acquired directly from diet, it is of interest to see to what degree they can serves as substrates for synthesis of very long chain fatty acids.

Docosapentaenoic and docosahexaenoic acid are strong candidates for the synthesis of very long chain fatty acids. Since these two fatty acids are generally considered to be the major end products of n-6 and n-3 fatty acid metabolism. Animals fed C22:6n-3 in the diet do not contain higher n-3 hexaenoic acid in the photoreceptor compared to animals fed a diet without C22:6n-3 (Chapter IV & V). Voss et al. (1991 & 1992) have reported an alternative pathway in liver such that C22:5n-3 and C22:6n-3 are produced by β-oxidation of C24:6n-3 which is desaturated from C24:5n-3, after elongation from C22:5n-3. This system is also functioning in the retina *in vitro* (Rotstein et al., 1996). In this case, it is interesting to ask questions whether C22:6n-3 is directly involved in further synthesis of very long chain fatty acids. If C22:6n-3 is not the substrate for very long chain fatty acids, than perhaps C20:5n-3 is the preferred substrate.

Dietary n-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid, can greatly affect the fatty acid composition of membrane phospholipids in nervous tissues within a relatively short time (Philbrick et al., 1987; Foot et al., 1982; Hargreaves & Clandinin, 1987a, 1987b & 1989c). Cellular elongation and the desaturation and deacylation- reacylation cycle in retina may partially depend on dietary fat availability. In this regard, long chain fatty acids (Anderson & Connor, 1994; Pawlosky et al., 1997) and very long chain fatty acids (Suh et al., 1994) in photoreceptor cells respond to the dietary n-6/n-3 fat fed. However, the involvement of diet in the synthesis of very long chain fatty acids has not been studied with practical diet fat intake levels.

The structural and functional characteristics of retinas are sensitive to the light exposure. Light elicits the release of arachidonic acid (Birkle & Bazan, 1989; Jung & Reme, 1994) and docosahexaenoic acid (Reinboth et al., 1996) which is mediated by the activated form of phospholipase A_2. The fatty acids subsequently released produce eicosanoids and docosanoids (Reinboth et al., 1995; Birkle & Bazan, 1989; Bazan et al., 1984). The effect of light exposure on the synthesis of very long chain fatty acids is not known.

Therefore, the objective of this experiment was to characterize incorporation and distribution of C20:5n-3 and C22:6n-3 into long chain and very long chain fatty acids in phospholipids of rat retina, to determine the effect of dietary fat on the synthesis of very long chain fatty acids from these substrates and to assess the effect of light exposure on the metabolism of these fatty acids in the retina.

MATERIALS AND METHODS

Animals and Diets

One hundred and forty four male weanling Sprague-Dawley rats were obtained from the University of Alberta Vivarium. Animals were housed under cyclic light with 12 hour dark and 12 hour light. The intensity of illumination at the front of the cage was 110 lux (1.52 Quantum, μEm^2sec^{-1}) and at the centre of the cage was 27 lux (0.38 Quantum, μEm^2sec^{-1}) of cool, white fluorescent light. Animals were randomly assigned to two experimental groups and fed a semipurified diet containing 20% (w/w) fat. Each diet provided 1% (w/w) of fat as C22:6n-3 (docosahexaenoic acid, DHA) or C18:3n-3 (linolenic acid, LNA). The fatty acid composition of experimental diets fed is illustrated (Table VIII-1). Diets were prepared weekly and kept at -30°C until fed. Body weight was taken at the start (weanling weight, 52.2 ± 13.6g) and after 50 days of diet treatment (522 ± 52 g). After 6 weeks of feeding, animals in each diet group were anesthesized with halothane and injected intravitreally into each eye with either 1 μCi [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 (^{3}H-20:5n-3, specific activity, 150 Ci/mmol,

Table VIII-1. Fatty acid composition of experimental diets.

Diet	DHA[a]	LNA[b]
Fatty acid (% w/w)		
C14:0	2.4	1.9
C16:0	22.1	21.8
C18:0	50.8	50.9
C18:1n-9+n-7	4.4	4.6
C18:2n-6	15.6	16.2
C18:3n-3	0.1	1.1
C20:4n-6	0.1	-
C20:5n-3	0.0	-
C22:6n-3	1.0	-
ΣSAT[c]	78.3	77.5
ΣMONO[d]	4.8	5.0
Σn-6[e]	15.7	16.2
Σn-3[f]	1.1	1.2
P/S ratio[g]	0.22	0.23

Fatty acids with chain length greater than C24 were present less than 0.05% (w/w) in both diets.[a]DHA, docosahexaenoic acid; [b]LNA, linolenic acid; [c]ΣSAT, sum of saturated fatty acids; [d]Σ Mono, sum of mono unsaturated fatty acids; [e]Σn-6, sum of n-6 fatty acids; [f]Σn-3, sum of n-3 fatty acids; [g]P/S ratio, polyunsaturated to saturated fatty acid ratio

American Radiolabeled Chemicals, St. Louis, MO) or [4,5-^{3}H]22:6n-3 (^{3}H-22:6n-3, specific activity, 60 Ci/mmol, New England Nuclear-DuPont, Boston, MA) in 2 µl ethanol using a 32 gauge needle. Animals were then transferred to polypropylene cages and maintained in either continuous light at the intensity of 1100 lux (15 Quantum, µEm^{2}sec^{-1}) or complete darkness for 48 hours.

Isolation of Photoreceptor and Cell Body from the Retina

Animals were decapitated and retinas were isolated either in dim red light for dark adapted animals or in ambient laboratory light for light exposed groups. All isolations were performed at 4°C. Eight to ten retinas were pooled for the separation of the rod photoreceptor and the cell body by discontinuous sucrose gradient ultra centrifugation (Stinson et al., 1991a). Fresh samples were homogenized in 0.5 ml or 1 ml of 50 mM tris-acetate buffer (pH 7.4) containing 5 mM $MgCl_2$ and 0.1mM EGTA for photoreceptor or the cell body, respectively. An aliquot (10 ul) of each sample was taken for protein analysis using bovine serum albumin as standard (Lowry et al., 1951).

Lipid Analysis

Lipids were extracted from the photoreceptor or cell body according to the procedure of Folch et al. (1957). The lipid extract was dried under nitrogen and dissolved in 100 µl chloroform. An aliquot was then taken and counted for measurement of incorporation of radioactivity into the total lipid of each cell fraction. Twenty percent of the remaining lipid extract was used to determine the distribution of radioactivity into individual phospholipids and neutral lipids in the photoreceptor and cell body. Individual phospholipid classes were isolated by the method described previously (Suh et al., 1994). After scraping off phospholipids into 20 ml scintillation vials, the silica gel containing neutral lipid was also scraped. To allow the elution of lipids from the silica gel, vials containing 0.5 ml of water were kept for 5 hours at 45°C before adding 15 ml of scintillation cocktail (ScintiSafe™ Econo 1, Fisher Scientific, Fair-Lawn, New Jersey, USA). Samples were counted in a Beckman LS-5801 liquid scintillation spectrometer (Irvine, CA, USA). The total lipid extract was also separated into individual phospholipid classes and then fatty acid methyl esters were prepared with 14% (w/w) boron trifluoride-methanol reagent (Morrison and Smith, 1964).

Analysis of Fatty Acid Methyl Esters

For analysis of the labeled fatty acids present in each phospholipid, two samples containing combined photoreceptor and cell body were pooled to ensure enough radioactivity for further fatty acid analysis. After pooling, an aliquot of each sample was analyzed by GLC to obtain a chromatogram with the precise retention time of each peak. A Hewlett Packard 5890 GLC equipped with a flame ionization detector MS ChemStation (HP-UX series) data system was used. The chromatography utilized a polar fused silica BPX 70 column (25 m x 0.2 mm I.D.). Helium was used as the carrier gas at a flow rate of 1.2 ml/min with a splitless injection. To maximize resolution and allow enough time for the collection of radiolabeled fatty acids, the oven temperature was programmed as follows: The initial oven temperature was 90°C, increased to 287°C at a rate of 3°C/min and held for 5 min for a total running time of 88 min. Degree of unsaturation and n-series of VLCFA were confirmed by GC-MS (Suh et al., 1994). The content of very long chain fatty acids obtained was not corrected for detector response factors, since the standards for these fatty acids are not available. A simple scheme of the experimental procedures is illustrated in Figure VIII-1.

Separation of Fatty Acids by Argentation TLC

The above GLC conditions resolved the very long chain fatty acids. However, C22:5n-3 cochromatographed with C22:6n-3 because of the high content of C22:6n-3 when 16 to 20 retinas were pooled. Therefore, argentation TLC was applied to separate fatty acids on the basis of degree of unsaturation to avoid this overlap (see Appendix Figure A-4). Silica gel-H plates were impregnated with 12% (w/v) $AgNO_3$ and developed

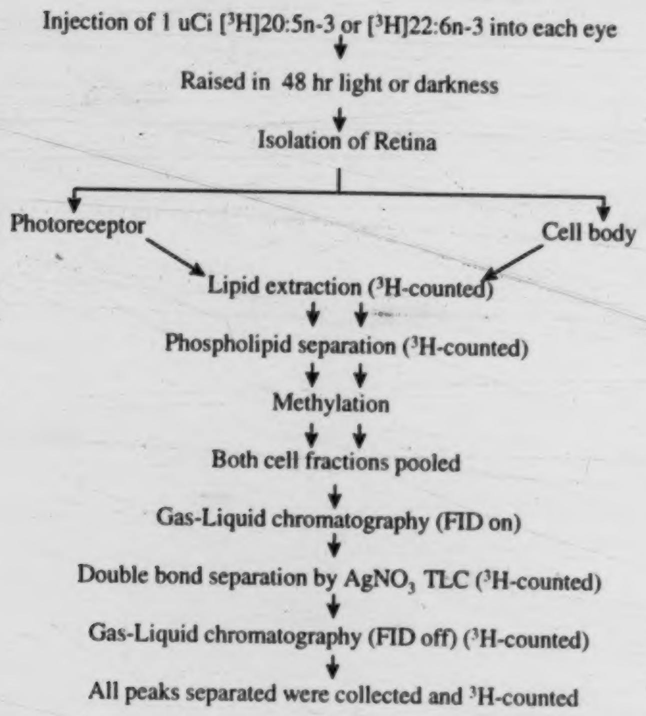
Injection of 1 uCi [³H]20:5n-3 or [³H]22:6n-3 into each eye
Raised in 48 hr light or darkness
Isolation of Retina
Photoreceptor
Cell body
Lipid extraction (³H-counted)
Phospholipid separation (³H-counted)
Methylation
Both cell fractions pooled
Gas-Liquid chromatography (FID on)
Double bond separation by AgNO₃ TLC (³H-counted)
Gas-Liquid chromatography (FID off) (³H-counted)
All peaks separated were collected and ³H-counted

Figure VIII-1. Simple scheme of the procedures.

three times in a developing system of hexane:diethyl ether:acetic acid:toluene:acetone (50:4:2:40:4, by vol) for 40 min each development. After visualizing bands with u.v. light by spraying with 0.1% (w/v) 2'7'-dichlorofluorescein in 95% (w/v) ethanol, bands containing a different number of double bonds were separated into 3 major fractions (I, II, III) to save time during collection of radiolabeled fatty acid methyl esters in GLC. These fractions contained the following fatty acids; (I), saturated, mono and dienoic fatty acids; (II), tri and tetraenoic fatty acids, pentaenoic long and very long chain fatty acids (C24:5-C36:5) and hexaenoic very long chain fatty acids (C28:6-C36:6); (III), C22:6n-3, C24:6n-3 and C26:6n-3. Fatty acids separated by GLC from band II and band III are indicated in Figure VIII-2. The peak overlap between C22:5n-3 and C22:6n-3 on GLC was avoided by this procedure. Each group of fatty acids was eluted in two steps. The first step of elution

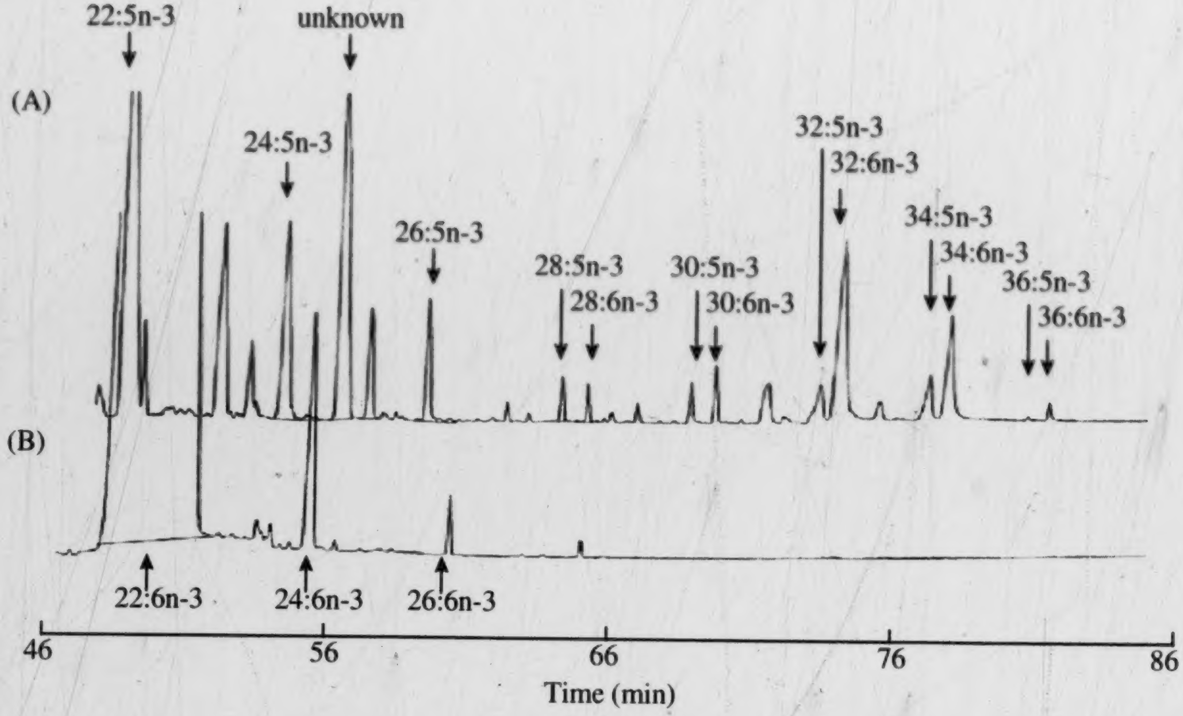

Figure VIII-2. Chromatograms illustrating polyenoic fatty acid methyl esters of phosphatidylcholine in the retina after resolution by argentation TLC. Fatty acid methyl esters were separated by double bonds using $AgNO_3$ TLC and collected into only 3 fractions (I, II, III) to save time during collection in gas chromatography. The peak overlap between C22:5n-3 and C22:6n-3 is avoided by this procedure. Only n-3 fatty acids are indicated by the arrows. (A) represents the fatty acid components in fraction II and (B) fraction III. Fraction I containing saturates, monoenes and dienes is not shown. These fatty acids were separated by gas-liquid chromatography using a BPX 70 column with running time 88 min. A peak indicated by unknown is a contaminated peak from elution.

used the following solvent system; chloroform:methanol:acetic acid:water (50:39:1:10, by vol) (Arvidson, 1968). Test tubes containing each band and the solvent system were located in a sonic water bath for 10 min and this step was repeated 3 times. The combined extracts were partitioned with 4N NH_4OH and washed with methanol: 1% NaCl (1:1, v/v). The second elution was effected by methylation of the remaining silica gel with 14% (w/w) boron trifluoride-methanol. The first elution recovered more C22:6n-3 and very long chain fatty acid of C30, C34 and C36 and the second elution more very long chain fatty acid of C24, C26 ,C28 and C32. The resulting extracts were combined and dried under nitrogen and then dissolved in 100 µl hexane. An aliquot was taken to count the distribution of radioactivity between double bond fractions of fatty acid methyl esters.

Collections of Radiolabeled Fatty Acid Methyl Esters

Radiolabeled fatty acid methyl esters from argentation fractions, II and III, were separated by gas-liquid chromatography as mentioned above except the flame ionization detector was not ignited. The elution conditions allowed enough time for collection of each peak manually. The trapping device used was an inverted Pasteur pipette packed with wet glass wool at the upper part of the pipette. To avoid the leakage of radioactivity at the bottom of the pipette slight vacuum was applied at the top. With this system, radioactivity collected on the inner surface wall of pipette around the glass wool. This procedure was found to be appropriate to trap low counts of radioactivity. Fraction II contained radiolabeled C18:3n-3, C20:5n-3, C22:5n-3, C24:5n-3, C26:5n-3, C28:5n-3, C28:6n-3, C30:5n-3, C30:6n-3, C32:5n-3, C32:6n-3, C34:5n-6, C34:6n-3, C36:5n-3 and C36:6n-3. Fraction III contained C22:6n-3, C24:6n-3 and C26:6n-3 (Figure VIII-2). Two background peaks were also collected for 2 min before (20 min-22 min) and after (85 min-87 min) peak collections. After collection the pipette was cut into 5 pieces and added into scintillation vials.

Radioactivity was determined in a Beckman LS 9800 liquid scintillation spectrometer (Irvine, CA, USA). Samples contained Pasteur pipette, wet glass wool and

20 ml of scintillation cocktail (Formula-963, Packard, The Netherlands) in a 20 ml plastic scintillation vials. Each sample count was corrected for background (pipette, glass wool, scintillation fluid and vials), representing the same period of time for which the sample peak was collected. The counts less than the background plus the 2 S.D. of background counts were eliminated as background noise.

Statistical Analysis

The effect of isotope, sample fractions, light and diet on the protein, phospholipids and the fatty acid composition of phospholipids were analyzed by analysis of variance of a split-plot design. After pooling photoreceptor and cell body for the analysis of radiolabeled fatty acid methyl esters, three way-analysis of variance was used to test the effect of isotope, light and diet. The analysis of radioactivity distribution in fractions after argentation TLC used one way analysis of variance to test for a difference between substrates injected, ^{3}H-20:5n-3 and ^{3}H-22:6n-3. A Duncan's multiple range test (Steel & Torrie, 1990) was used for comparision of the main factors. All data is expressed as mean ± standard error of the mean (S.E.M.).

RESULTS

Incorporation of Labeled C20:5n-3 or C22:6n-3 into Retinal Cell Fractions

In vivo incorporation of ^{3}H-20:5n-3 and ^{3}H-22:6n-3 into lipids of photoreceptor and cell body of the retina was studied after feeding n-3 fatty acids in an environment of continuous light. The photoreceptor lost a significant amount of its protein by 48 hour light exposure in both LNA (53.4%, w/w) and DHA (64.1%, w/w) fed groups regardless of radioactive substrates injected (Figure VIII-3). In the cell body, only the LNA fed group injected with ^{3}H-20:5n-3 lost its protein (34.0%, w/w) after the light treatment. However, light significantly increased the incorporation of ^{3}H-20:5n-3 into total lipid of the photoreceptor and the cell body expressed as dpm x 10^3 per mg protein in groups fed the LNA diet (Figure VIII-4). When the incorporation of ^{3}H-20:5n-3 and ^{3}H-22:6n-3 into total lipid of both cell fractions was compared in rats fed a LNA diet and exposed to light, the former incorporated more substrate into the cell body (60.6%) than into the photoreceptor (39.4%) and the latter incorporated more label into the photoreceptor (63.4%) than into the cell body (36.6%) (Figure VIII-5).

Incorporation of Labeled C20:5n-3 or C22:6n-3 into Phospholipids

After metabolism of ^{3}H-20:5n-3 *in vivo* for 48 hours, the radioactivity in phospholipid in the photoreceptor and cell body was 0.12% and 2.48% of total amounts injected, respectively. The radioactivity from ^{3}H-22:6n-3 was 0.06% and 1.22%, respectively. There was no significant difference in the pmol level between ^{3}H-20:5n-3

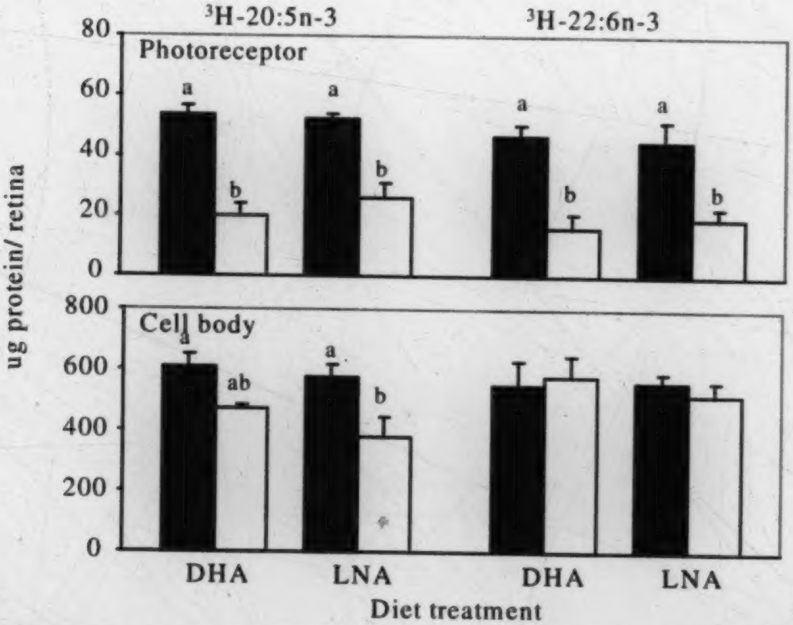

Figure VIII-3. Effect of dietary fat and light exposure on protein content in the photoreceptor and cell body of retinas injected with [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 and [4,5-^{3}H]22:6n-3. The values are the mean (n=4) ± S.E.M. Eight to ten retinas were pooled for each n. Values without a common letter within an isotope in each retinal fraction are significantly different, $p<0.05$. Significant effects were identified by analysis of variance of a split-plot design for light ($p<0.005$) and cell fraction ($p<0.0001$). No significant effects of diet or substrate were identified. DHA, docosahexaenoic acid; LNA,Linolenic acid. ■, dark; ☐, light.

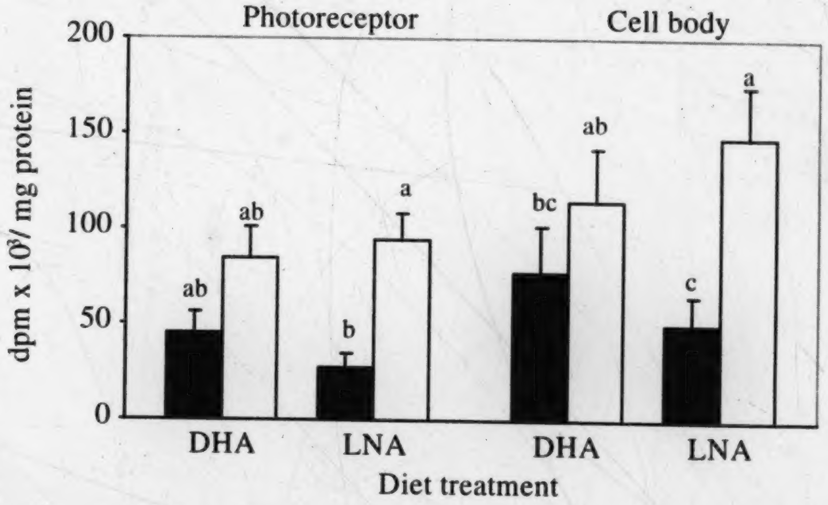

Figure VIII-4. Effect of dietary fat and light exposure on incorporation of [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 into total lipid in the photoreceptor and cell body of the retina. The values are the mean (n=4) ± S.E.M. Eight to ten retinas were pooled for each n. Values without a common letter within a retinal fraction are significantly different, $p<0.05$. Significant effects were identified by analysis of variance of a split-plot design for light ($p<0.003$) and cell fraction ($p<0.006$). No significant effects of diet were identified. DHA, docosahexaenoic acid; LNA, Linolenic acid. ■, dark; □, light.

and ^{3}H-22:6n-3 in the phospholipid of each cell fraction (0.05 pmol and 0.08 pmol, respectively in the photoreceptor and 1.19 pmol and 1.55 pmol, respectively in the cell body). Since the incorporation of ^{3}H-20:5n-3 into phospholipid was twice as much as for ^{3}H-22:6n-3 in both fractions, the data in Figure VIII-6 is expressed as % distribution of substrate in individual phospholipid.

Most radioactivity was found in the major phospholipids comprising phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (88% of ^{3}H-20:5n-3 and 90% of ^{3}H-22:6n-3) compared to phosphatidic acid (6.2%) and neutral lipids (4.8%) (Figure VIII-6). The incorporation pattern of ^{3}H-20:5n-3 into individual phospholipids was distinct ($p<0.0005$) compared to ^{3}H-22:6n-3 for both cell fractions of the retina regardless of diet and light treatment. In the photoreceptor, overall % of radioactivity from ^{3}H-20:5n-3 was 29% in phosphatidylcholine, followed by phosphatidylserine and phosphatidylethanolamine (26.5% and 26.4%, respectively). However in the cell body, ^{3}H-20:5n-3 was incorporated more into phosphatidylethanolamine (38%) compared to phosphatidylcholine (26.5%). ^{3}H-22:6n-3 was preferentially incorporated into phosphatidylethanolamine in both cell fractions of the retina (Figure VIII-6). When the incorporation of the label was compared between cell fractions, sphingomyelin and phosphatidylcholine contained higher amounts of incorporation from both substrates in the photoreceptor whereas incorporation into phosphatidylethanolamine is greater in the cell body (Figure VIII-6).

Incorporation of Labeled C20:5n-3 or C22:6n-3 into Fatty Acid Separated on Argentation TLC

After measuring the radioactivity distribution in the individual phospholipids, two samples were pooled after combining the photoreceptor and the cell body. The % distribution of radioactivity between fatty acid double bonds was not significantly

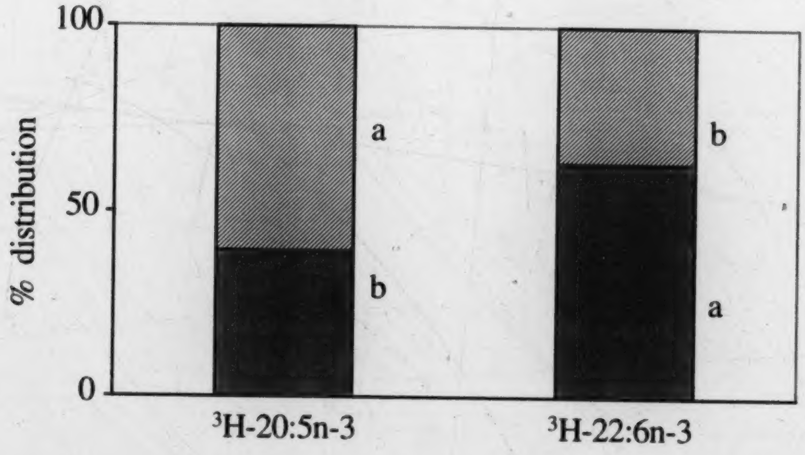

Figure VIII-5. Incorporation of [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 and [4,5-^{3}H]22:6n-3 into total lipid in the photoreceptor and cell body of the retina from rats fed a linolenic acid diet and exposed to light. The values are the mean (n=4) ± S.E.M. Eight to ten retinas were pooled for each n. Values without a common letter are significantly different, $p<0.025$. ■, photoreceptor; ▨, cell body.

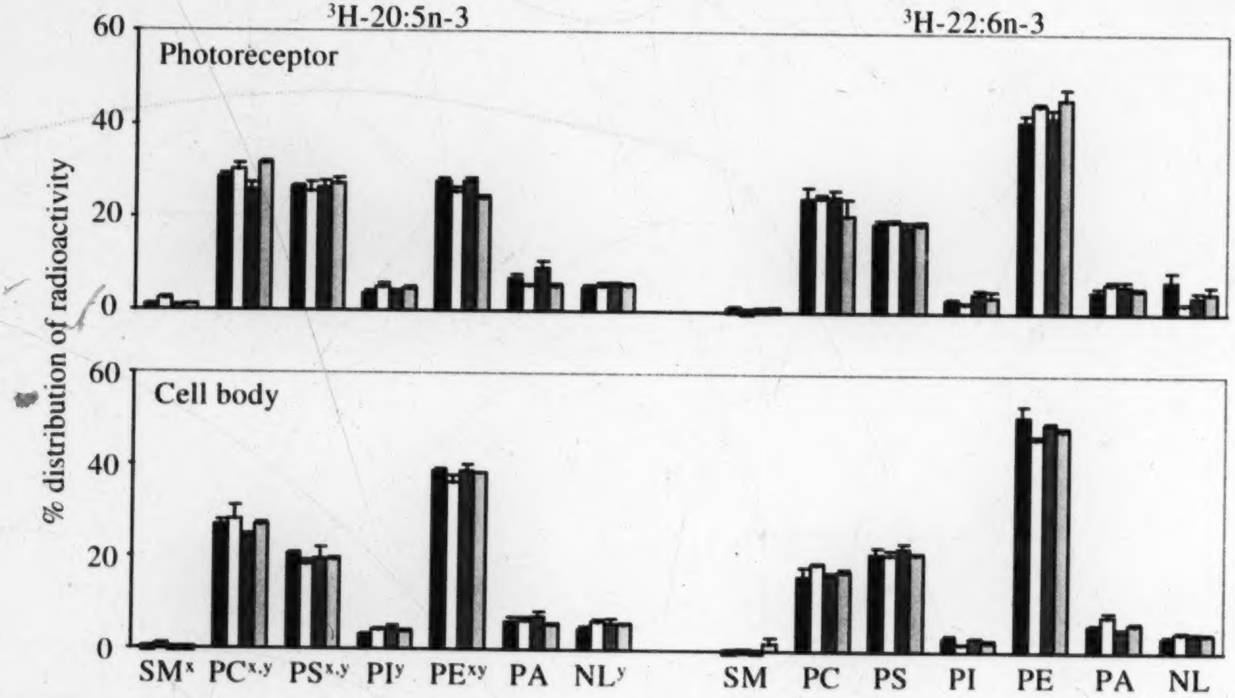

Figure VIII-6. Incorporation of [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 and [4,5-^{3}H]22:6n-3 into phospholipids and neutral lipids in the photoreceptor and cell body of the retina following diet and light treatment. The values are the mean (n=4) ± S.E.M. Eight to ten retinas were pooled for each n. Values without a common letter within a phospholipid class were significantly different, $p<0.05$. Superscripts indicated for each legend represent significant effect of cell fractions (^{x}, $p<0.005$) or substrates (^{y}, $p<0.0005$). No significant effects of diet or light were identified. SM, sphingomyline; PC, phosphatidylcholine: PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; NL, neutral lipids. ■, DHA diet/dark; □, DHA diet/light; ▩, LNA diet/dark; ▨, LNA diet/light.

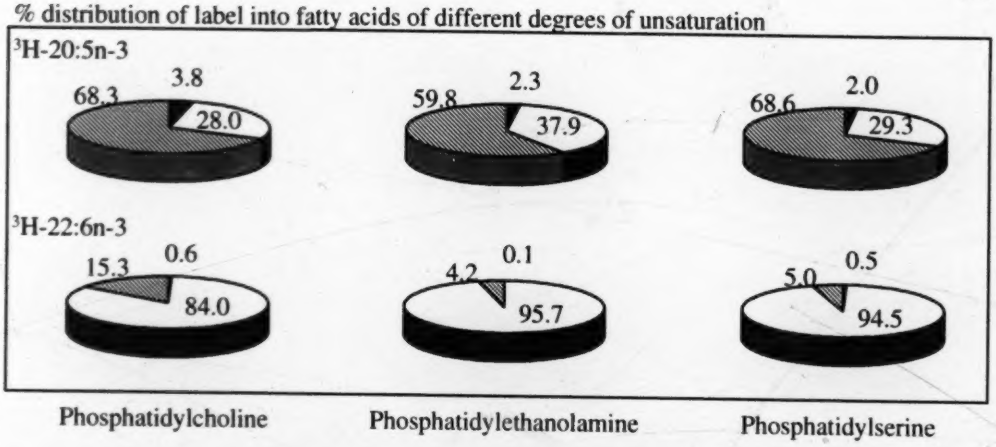

Figure VIII-7. Incorporation of [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 and [4,5-^{3}H]22:6n-3 into fatty acids of the retina after argentation TLC. In each phospholipid two samples containing combined photoreceptor and cell body were pooled. Fatty acid methyl esters were then separated by double bonds using $AgNO_3$ TLC and collected into only 3 fractions (I, II, III) to save time during collection in gas chromatography (Figure VIII-2) : (I) ■, saturated, mono and dienoic fatty acids; (II) ▨, tri and tetraenoic fatty acids, pentaenoic long and very long chain fatty acids (C24:5-C36:5) and hexaenoic very long chain fatty acids (C28:6-C36:6); (III) □, C22:6n-3, C24:6n-3 and C26:6n-3. The % distribution of radioactivity between double bonds were not significantly different between diet or light treatments. Therefore, the values represent a mean of 8 samples. Sixteen to twenty retinas were pooled for each n. Significant effects of substrate were identified ($p<0.0001$) in a class of phospholipids. Total label (pmol) of ^{3}H-20:5n-3 and ^{3}H-22:6n-3 in phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, respectively, is as follows:^{3}H-20:5n-3, 0.12, 0.17, 0.07; ^{3}H-22:6n-3, 0.18, 0.52, 0.22.

different between diet or light treatments. Significant effects of substrate were identified ($p<0.0001$), showing a different behavior for the two substrates as precursors of metabolites further down the pathway (Figure VIII-7). Small amounts of the label from ^{3}H-20:5n-3 were found in fraction I (3.8%) containing saturated, monoenes and dienes. Considerable amounts of the label from this substrate were found in fraction II (68.3%) containing tri and tetraenoic fatty acids, pentaenoic long and very long chain fatty acids (C24:5-C36:5) and hexaenoic very long chain fatty acids (C28:6-C36:6). Twenty- eight % of the label occurred in fraction III containing C22:6n-3, C24:6n-3 and C26:6n-3. Similar distribution of this substrate was found in phosphatidylethanolamine and phosphatidylserine. Most (84%-96%) of the ^{3}H-22:6n-3 was distributed into hexaenes of C22, C24 and C26 (fraction III) in the these major phospholipids (Figure VIII-7).

Incorporation of Labeled C20:5n-3 or C22:6n-3 into Fatty Acids

Typical percentage incorporation of ^{3}H-20:5n-3 and ^{3}H-22:6n-3 into n-3 fatty acids in the retina is illustrated (Figure VIII-8). The metabolic fate of the two substrates used was different. For example, among the fatty acid metabolites from ^{3}H-20:5n-3, pentaenoic long and very long chain fatty acids were the most actively metabolized and incorporated into phosphatidylcholine (63% and 19% respectively). The production of hexaenoic long and very long chain fatty acid was relatively small, 11% and 6%

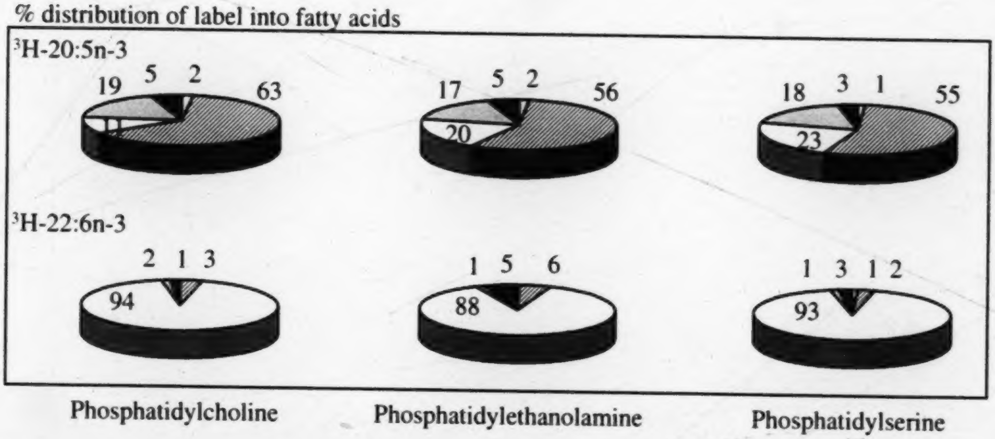

Figure VIII- 8. Typical percent incorporation of [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 and [4,5-^{3}H]22:6n-3 into n-3 fatty acids in the retina. Radioactive n-3 fatty acid methyl esters from argentation fraction II and III (Figure VIII-7.) were separated by gas chromatography (Figure VIII-2). Each peak was manually collected. The values represent the mean of 8 samples regardless of diet and light treatment to identify a typical pattern of incorporation. Sixteen to twenty retinas were pooled for each n. Significant effects of substrate were identified ($p<0.0001$) in a class of phospholipids. Detailed values for diet and light treatments are illustrated in Table VIII-2-VII-4 and Figure VIII-8. ☐ , n-3 trienoic long chain fatty acid (C18); ▨, n-3 pentaenoic long chain fatty acids (C20-C22); ☐, n-3 hexaenoic long chain fatty acid (C22); ▒, n-3 pentaenoic very long chain fatty acids (C24-C36); ■, n-3 hexaenoic very long chain fatty acids (C24-C36). Total label (pmol) of ^{3}H-20:5n-3 and ^{3}H-22:6n-3 in fatty acids of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, respectively, is as follows: ^{3}H-20:5n-3, 0.015, 0.014, 0.011; ^{3}H-22:6n-3, 0.030, 0.050, 0.051.

respectively (Figure VIII-8). Over 80% of the pentaenes are produced from ^{3}H-20:5n-3, illustrating the active metabolism of C20:5n-3. In contrast with ^{3}H-20:5n-3, over 90% of ^{3}H-22:6n-3 was not metabolized further by chain elongation. The product of ^{3}H-22:6n-3 retroconversion to pentaenes was only 4% and the elongated product of pentaenoic and hexaenoic very long chain fatty acid was 4% and 2% respectively. The same over all pattern of incorporation for each substrate was observed in phosphatidylethanolamine and phosphatidylserine (Figure VIII-8).

Effect of Dietary Fat and Light on Incorporation of Labeled C20:5n-3 or C22:6n-3 into Fatty Acids

Retinas were injected with ^{3}H-20:5n-3 and allowed to metabolize the label for 48 hours in the dark *in vivo*. The pattern of incorporation into individual fatty acids was significantly different in phosphatidylcholine. The radiolabeled fatty acids were found in carbon chain length up to C34, indicating that C20:5n-3 was the precursor of metabolites. Over 35% to 40% of ^{3}H-20:5n-3 was elongated to C22:5n-3 and then C22:6n-3 and C24:5n-3 (Table VIII-2). Small portions of ^{3}H-20:5n-3 was retroconverted to C18:3n-3. Among the major phospholipids in the retina, phosphatidylcholine compared to other phospholipids contained the highest amount of C20:5n-3 (Table VIII-2; VIII-3; VIII-4). However over 94% of ^{3}H-22:6n-3 in phosphatidylcholine was not involved in further metabolism. The level of retroconverted product, C22:5n-3, was minor. Among ^{3}H-20:5n-3 metabolites, the production of long chain pentaenes and hexaenes was highest in animals fed DHA. Conversely the LNA group exhibited higher amounts of incorporation into very long chain pentaenoic and hexaenoic fatty acids (Table VIII-2; Figure VIII-9) into phosphatidylcholine. Specially the radioactivity in C26:5, C24:6 and C30:6 from ^{3}H-20:5n-3 was higher in animals fed a LNA diet compared to animals fed the DHA diet. Radioactivity of C24:5 and C24:6 from ^{3}H-22:6n-3 is higher in animals fed the LNA diet compared to animals fed DHA diet. The ratio between labeled C24:5n-3 and C22:6n-3 was 1.02 and 0.15 from ^{3}H-20:5n-3. These suggest that 1.02 mol of C24:5n-3 and 0.15 mol of C24:6n-3 is formed for every 1 mol of ^{3}H-20:5n-3 metabolized (Table VIII-2).

For ^{3}H-22:6n-3, the ratio was only 0.01and 0.02, respectively. This illustrates that a much higher proportion of C24:5 and C24:6 was produced ^{3}H-20:5n-3 by elongation and desaturation than from ^{3}H-22:6n-3.

Table VIII-2. Incorporation of [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 and [4,5-^{3}H]22:6n-3 into n-3 long and very long chain fatty acids (C24-C36) in phosphatidylcholine from retinas of dark adapted rats following dietary fat treatment.

Isotope	^{3}H-20:5n-3		^{3}H-22:6n-3			
Diet	DHA	LNA	DHA	LNA		
Distribution (%)					Pooled SEM	Diet
n-3 LCFA						
C18:3	1.3	2.8	0.0	0.0	0.6	
C20:5	25.9^{a}	21.1^{b}	0.0^{c}	0.0^{c}	0.6	***
C22:5	39.8	36.6	3.3	1.3	2.5	
C22:6	13.5	11.3	96.7	94.4	0.9	
n-3 pentaenoic VLCFA						
C24:5	10.3	12.8	0.0	1.1	0.9	
C26:5	3.0^{b}	7.3^{a}	0.0^{c}	0.7^{c}	0.5	***
C28:5	1.5	2.0	0.0	0.0	0.1	
C30:5	0.8	1.1	0.0	0.0	0.5	
C32:5	0.6	1.4	0.0	0.0	0.3	
C34:5	0.3	0.0	0.0	0.0	0.2	
C36:5	0.0	0.0	0.0	0.0	0.1	
n-3 hexaenoic VLCFA						
C24:6	1.0^{bc}	2.6^{a}	0.0^{c}	1.5^{b}	0.3	***
C26:6	0.6	1.2	0.0	0.6	0.3	
C28:6	0.8	1.5	0.0	0.3	0.3	
C30:6	0.6^{b}	0.7^{a}	0.0^{b}	0.0^{b}	0.1	***
C32:6	0.6	0.0	0.0	0.0	0.2	
C34:6	0.7	0.0	0.0	0.0	0.1	
C36:6	0.0	0.0	0.0	0.0	0.1	

Values given are means (n=2) and pooled S.E.M. is presented. For each n 16 to 20 retinas were pooled. Rats were injected intravitreally into each eye with either 1 µCi [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 (specific activity, 150 Ci/mmol) or [4,5-^{3}H]22:6n-3 (specific activity, 60 Ci/mmol) and maintained in either continuous light (15 Quantum, $\mu Em^{2}sec^{-1}$) or complete darkness for 48 hours. Lipid was extracted and radiolabeled n-3 fatty acid methyl esters were separated by gas chromatography (Figure VIII-2) after separating on argentation TLC (Figure VIII-7). Each peak was manually collected. Significant effects of substrate were identified ($p<0.03$). Values without a common superscript are significantly different between diets (***, $p<0.001$). LCFA, long chain fatty acids; VLCFA, very long chain fatty acids. Variable for which no value (zero) is given did not exhibit counts greater than background + 2 S.D. Total label (pmol) of ^{3}H-20:5n-3 and ^{3}H-22:6n-3 in fatty acids of DHA or LNA, respectively, is as follows: ^{3}H-20:5n-3, 0.016, 0.008; ^{3}H-22:6n-3, 0.011, 0.059.

The incorporation of label from ^{3}H-20:5n-3 into C22:6n-3 in the dark decreased 30% after light exposure of animals fed the DHA containing diet (Figure VIII-9). The incorporation of label from ^{3}H-20:5n-3 into C22:6n-3 in animals fed the LNA diet was also decreased 12% by light treatment. Light exposure also decreased the production of total pentaenoic very long chain fatty acids in both DHA or LNA fed animals (9% and 3% respectively). The same significant effects of light were also observed on the incorporation of label from ^{3}H-20:5n-3 into C22:6n-3 of phosphatidylserine in animals fed either diet (data not shown).

In both phosphatidylethanolamine and phosphatidylserine, radiolabeled fatty acids were found in carbon chains up to C26 (Table VIII-3; VIII-4). The major fatty acid labeled from ^{3}H-20:5n-3 in phosphatidylethanolamine was C22:5n-3 followed by C22:6n-

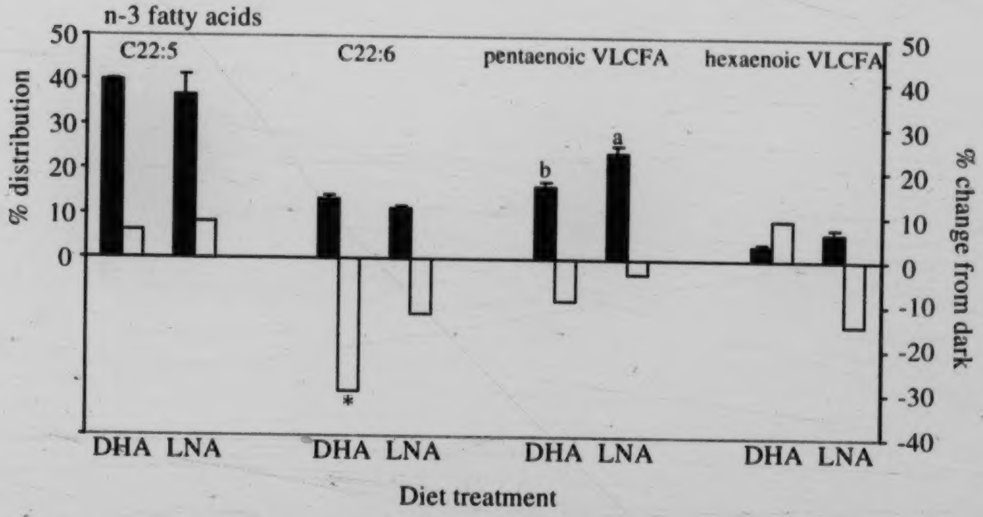

Figure VIII-9. Effect of dietary fat and light exposure on the distribution of n-3 long chain fatty acids and total n-3 pentaenoic and hexaenoic very long chain fatty acids (C24-C36) in phosphatidylcholine of retinas injected with [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3. The values for the dark adapted group are the mean (n=2) ± S.E.M. Values without a common letter in pentaenoic very long chain fatty acid is significantly different, $p<0.002$. Significant effect of light was identified in C22:6 of retina fed DHA diet, $p<0.03$. This effect was also found in C22:6 in phosphatidylserine on both diets (data not shown). Sixteen to twenty retinas were pooled for each n. The value of % change from dark by light treatment was taken from the total mean of each group. ■, dark; □, light.

3 and C24:5n-3. Over 89% to 95% of ^{3}H-22:6n-3 was directly incorporated into C22:6n-3 in phosphatidylethanolamine. There were no differences in diet and light treatment on metabolites ^{3}H-20:5n-3 in phosphatidylethanolamine. Animals fed the DHA diet had more radioactivity in C22:5n-3 and C24:6n-3 decreased from ^{3}H-22:6n-3 compared to animals fed the LNA diet.

The major fatty acid incorporated into phosphatidylserine from ^{3}H-20:5n-3 was the same as that found in phosphatidylethanolamine (Table VIII-4). In both phosphatidylethanolamine and phosphatidylserine, ^{3}H-20:5n-3 was incorporated more into pentaenoic very long chain fatty acids than hexaenes.

Table VIII-3. Incorporation of [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 and [4,5-^{3}H]22:6n-3 into n-3 long and very long chain fatty acids (C24-C26) in phosphatidylethanolamine from retinas of dark adapted rats following dietary fat treatment.

Isotope	^{3}H-20:5n-3		^{3}H-22:6n-3		
Diet	DHA	LNA	DHA	LNA	
Distribution (%)					Pooled SEM
n-3 LCFA					
C18:3	2.5	0.6	0.4	0.0	0.8
C20:5	3.6	12.2	0.0	0.0	2.0
C22:5	55.0	50.1	7.4^{*}	2.6	3.0
C22:6	20.2	16.2	88.8^{*}	95.1	2.5
n-3 VLCFA					
C24:5	12.0	13.3	0.0	0.6	1.1
C26:5	4.5	5.1	1.1	0.2	1.2
C24:6	1.6	3.3	4.0^{*}	1.4	1.7
C26:6	2.8	1.7	2.3	0.3	0.8

Values given are means (n=2) and pooled S.E.M. is presented. For each n 16 to 20 retinas were pooled. Rats were injected intravitreally into each eye with either 1 μCi [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 (specific activity, 150 Ci/mmol) or [4,5-^{3}H]22:6n-3 (specific activity, 60 Ci/mmol) and maintained in either continuous light (15 Quantum, $\mu Em^{2}sec^{-1}$) or complete darkness for 48 hours. Lipid was extracted and radiolabeled n-3 fatty acid methyl esters were separated by gas chromatography (Figure VIII-2) after separating on argentation TLC (Figure VIII-7). Each peak was manually collected. Significant effects of substrate were identified ($p<0.03$). * represents a significant effect of diet within ^{3}H-22:6n-3 injected group, $p<0.02$. LCFA, long chain fatty acids; VLCFA, very long chain fatty acids. Variable for which no value (zero) is given did not exhibit counts greater than background + 2 S.D. Total label (pmol) of ^{3}H-20:5n-3 and ^{3}H-22:6n-3 in fatty acids of DHA or LNA, respectively, is as follows: ^{3}H-20:5n-3, 0.014, 0.010; ^{3}H-22:6n-3, 0.025, 0.107.

Table VIII-4. Incorporation of [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 and [4,5-^{3}H]22:6n-3 into n-3 long and very long chain fatty acids (C24-C26) in phosphatidylserine from retinas of dark adapted rats following dietary fat treatment.

Isotope	^{3}H-20:5n-3		^{3}H-22:6n-3			
Diet	DHA	LNA	DHA	LNA		
Distribution (%)					Pooled SEM	Diet
n-3 LCFA						
C18:3	0.4	1.0	1.5	0.5	0.4	
C20:5	0.8	0.8	2.0	0.6	0.8	
C22:5	51.0	56.6	3.0	0.9	1.7	
C22:6	27.1^b	24.6^{bc}	91.4^a	94.8^a	1.2	*
n-3 VLCFA						
C24:5	12.6	11.5	2.0	0.6	1.1	
C26:5	6.5	4.7	0.7	0.5	0.6	
C24:6	2.1	1.8	2.0	2.7	0.8	
C26:6	2.2	2.0	1.1	0.5	0.6	

Values given are means (n=2) and pooled S.E.M. is presented. For each n 16 to 20 retinas were pooled. Rats were injected intravitreally into each eye with either 1 µCi [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5n-3 (specific activity, 150 Ci/mmol) or [4,5-^{3}H]22:6n-3 (specific activity, 60 Ci/mmol) and maintained in either continuous light (15 Quantum, μEm^2sec^{-1}) or complete darkness for 48 hours. Lipid was extracted and radiolabeled n-3 fatty acid methyl esters were separated by gas chromatography (Figure VIII-2) after separating on argentation TLC (Figure VIII-7). Each peak was manually collected. Significant effects of substrate were identified ($p<0.0001$). Values without a common superscript are significantly different between diets (*, $p<0.05$). LCFA, long chain fatty acids; VLCFA, very long chain fatty acids. Total label (pmol) of ^{3}H-20:5n-3 and ^{3}H-22:6n-3 in fatty acids of DHA or LNA, respectively, is as follows: ^{3}H-20:5n-3, 0.012, 0.009; ^{3}H-22:6n-3, 0.023, 0.099.

Effect of Dietary Fat and Light on Fatty Acid Composition

The effects of diet and light treatment on the ratio of n-6 tetrene to pentaene and of n-3 pentaene to hexaene very long chain fatty acid was tested in phosphatidylcholine of the retina (Figure VIII-10). Animals fed the DHA diet exhibited a higher ratio of n-6 tetraene to pentaene fatty acid of in C32 and C34 in chain length. The ratio of n-3 pentaene to hexaene very long chain fatty acid of carbon chain length of C24-C34 is lower in retinas from animals fed the DHA diet compared to those fed LNA. This suggests that dietary DHA decreases n-6 pentaenes more than n-6 tetraenes and increases n-3 hexaenes more than n-3 pentaenes. The ratio of very long chain fatty acid of n-6 tetraene to pentaene and n-3 pentaene to hexaene were significantly increased by light exposure in both dietary fat treatment groups. Specifically the ratio of n-6 tetraene to pentaene was increased in C28, C30, C34 and ratio of n-3 pentaene to hexaene in C24, C26, C30, C32 fatty acids. Light exposure may activate formation of n-6 tetraenes and n-3 pentaenes compared to n-6 pentaenes and n-3 hexaenes, respectively.

DISCUSSION

Characteristic Incorporation of Radiolabeled C20:5n-3 and C22:6n-3 into Retinal Membrane Fractions

The present study clearly demonstrated that ^{3}H-20:5n-3 and ^{3}H-22:6n-3 were taken up by the photoreceptor and the cell body after intravitreal injection. Light enhanced incorporation of both ^{3}H-20:5n-3 and ^{3}H-22:6n-3 into total lipid of the photoreceptor and the cell body. These observations are supported by previous reports that light stimulates incorporation of labelled glycerol and inositol into retinal lipids (Anderson et al., 1985; Bazan & Bazan, 1976; Anderson & Hollyfiels, 1981). Thus, light modulates the metabolic event in the retina and stimulates active incorporation of fatty acids into retinal membrane. It was interesting to find that ^{3}H-20:5n-3 was incorporated more into total lipid of the cell body (60.6%) whereas ^{3}H-22:6n-3 was incorporated more into the photoreceptors (63.4%). This finding suggests that ^{3}H-22:6n-3 is selectively up taken by the photoreceptor (De Turco et al., 1991) and maybe associated with retinal function. This also suggests that the retina contains the mechanism for cell specific uptake of fatty acids.

Incorporation of Radiolabeled C20:5n-3 and C22:6n-3 into Retinal Phospholipid

After intravitreal injection, ^{3}H-20:5n-3 (88%) and ^{3}H-22:6n-3 (99%) are esterified into phospholipids whereas, the incorporation into phosphatidic acid and neutral lipid was minor. This pattern was also found in the frog retina incubated with ^{3}H-22:6n-3 (De Turco et al., 1991). Analysis of distribution of radioactivity among individual

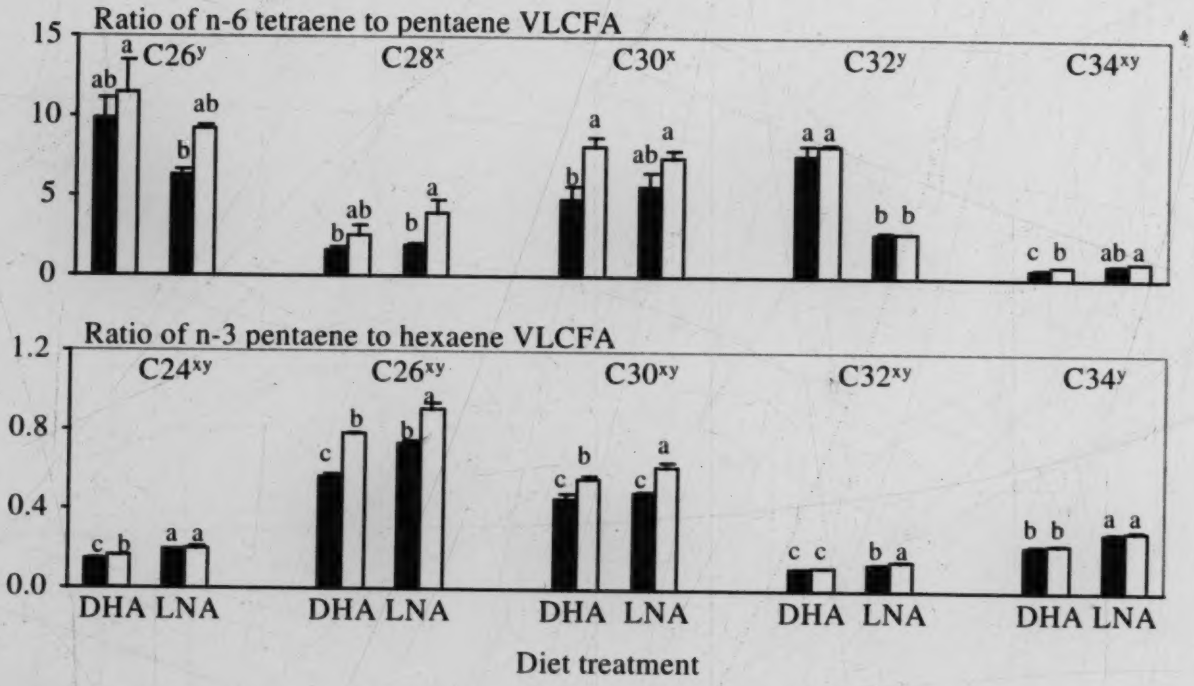

Figure VIII-10. Effect of dietary fat and light exposure on the ratio of n-6 tetraene to pentaene and of n-3 pentaene to hexaene very long chain fatty acid in phosphatidylcholine of the retina. Fatty acid methyl esters were injected into gas chromatography with flame ionization detector before resolved in argentation TLC. The samples from each substrate were pooled, therefore the values represent the mean (n=4) ± S.E.M. Sixteen to twenty retinas were pooled for each n. Values without a common letter within a fatty acid were significantly different, $p<0.05$. Superscripts for each chain length indicate differences as follows: significant effect of light (^{x}, $p<0.03$); significant effect of diet (^{y}, $p<0.03$). No interaction effects were identified. Where error bars are not evident, the error is too small to be illustrated. ■, dark; ☐, light.

phospholipids shows that ^{3}H-22:6n-3 is preferentially incorporated into phosphatidylethanolamine in both photoreceptor and cell body. Higher *in vivo* labeling of phosphatidylethanolamine from radiolabeled 22:6n-3 was also found in the rat (49% after 24 hours, Wetzel et al., 1991), frog (37% after 6 hours, De Tulco et al., 1994) and dog (49% after 24 hours, Wetzel et al., 1989) retinas. However, ^{3}H-20:5n-3 was incorporated equally into phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine in photoreceptors. In individual phospholipids in the cell body, ^{3}H-20:5n-3 was incorporated more into phosphatidylethanolamine. This unequal distribution of label among retinal phospholipids suggests that phospholipid has specificity for fatty acid types which may ultimately influence rhodopsin movement.

Incorporation of Radiolabeled C20:5n-3 and C22:6n-3 into Retinal Fatty Acids

Photoreceptor cells in the retina incorporated exogenous ^{3}H-20:5n-3 and ^{3}H-22:6n-3 into cellular lipids and actively desaturated and elongated them to longer chain fatty acids. Quite distinctive metabolism was identified from the incorporation pattern of ^{3}H-20:5n-3 and ^{3}H-22:6n-3. Over 88% to 90% of ^{3}H-22:6n-3 was incorporated directly into retinas without further metabolism in major phospholipids. Whereas, significant amounts of C22:5n-3 was produced from ^{3}H-20:5n-3 (38%, 53%, 54% in phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, respectively, regardless of diet) followed by C22:6n-3, C24:5n-3 and C24:6n-3. The significant accumulation of C22:5n-3 from intravitreally injected ^{14}C-18:3n-3 has also been found in total lipid of rat retinal debris (Wetzel et al., 1991). Although studies overlooked C22:5n-3, the highly selective elongation towards C22:5n-3 production may be an important metabolic step in the synthesis of docosahexaenoic acid as well as very long chain fatty acids in the retina.

The metabolic intermediates for production of C22:6n-3, C24:5n-3 and C24:6n-3, proposed by Voss et al. (1991) have been detected in this experiment. Rotstein et al. (1996) found the same intermediates in retina total lipid incubated with ^{14}C-22:5n-3. It is proposed that C24:5n-3 may give rise to longer pentaenes via elongation (Rotstein et al., 1996). The present experiment found that the synthesis of n-3 pentaenoic very long chain fatty acids up to C34 in phosphatidylcholine was favored more than synthesis of hexaenes from ^{3}H-20:5n-3. Thus, two different metabolic pathways are proposed (Figure VIII-11). One pathway uses C24:5n-3 as the substrate for successive elongation to n-3 pentaenoic and its desaturated product C24:6n-3 as the substrate for hexaenoic very long chain fatty acids, respectively. The precursors for this pathway are C20:5n-3 and C22:5n-3. The other pathway elongates each pentaenoic very long chain fatty acid as the substrate for synthesis of hexaenoic homologues by Δ^6 desaturation. It is reasonable to consider that if C24:5n-6 is the substrate for Δ^6 desaturation, then longer chain pentaenes can also be a substrate. The existence of different Δ^6 desaturases has been proposed in human Y-79 retinoblastoma and Jurkat T-cells, which is different from the classical enzyme responsible for the conversion of C18:3n-3 to C18:4n-3 (Marzo et al., 1996). Because the cellular location for synthesis of very long chain fatty acid is not known, the answer of whether this enzyme is located in the peroxisome or the microsome will enlighten many questions concerning synthesis of very long chain fatty acids. It is evident that the retina possesses active desaturase and elongase enzymes and is able to produce very long chain fatty acids.

Unlike the ^{3}H-20:5n-3, 90% of intravitreally injected ^{3}H-22:6n-3 was incorporated into phospholipids of retina without change. This implies that retina has a mechanism for selective uptake of C22:6n-3 through direct acylation. Many studies have implied that docosahexaenoic acid was preferentially taken up by retina (Wang et al., 1992; De Turco et al., 1990 & 1991), turnover and utilization for synthesis of new disc membrane (De Turco et al., 1990; Gordon & Bazan, 1990). Docosahexaenoic acid may be involved in modulation of docosahexaenoic containing molecular species and thereby retinal function which require higher concentration of this fatty acid.

Effect of Dietary Fat and Light on the Incorporation of Radiolabeled C20:5n-3 and C22:6n-3 into Retinal Fatty Acids

Feeding diet containing DHA tended to increase the incorporation of ^{3}H-20:5n-3 into n-3 pentaenoic and hexaenoic long chain fatty acids but decrease incorporation into very long chain fatty acid in phosphatidylcholine compared to feeding the diet containing LNA. Total pentaenoic very long chain fatty acids were significantly increased in phosphatidylcholine after feeding rats with the LNA diet. Although the exact implication of this finding is not known, the change in fatty acid composition is involved in rhodopsin function (Chapter V & VI).

The present study demonstrated light induced changes in long and very long chain fatty acid. The incorporation of label of ^{3}H-20:5n-3 into C22:6n-3 decreased significantly after light exposure in phosphatidylcholine and phosphatidylserine (data not shown). The incorporation of label from ^{3}H-22:6n-3 in C22:6n-3 was also decreased in phosphatidylcholine and phosphatidylethanolamine. This effect was more distinct with feeding of a diet containing DHA. This data is consistent with previous studies showing that light induced release of docosahexaenoic acid (Reinboth et al, 1996; Penn & Anderson, 1987) which may relate to rhodopsin bleaching and regeneration. The incorporation of radioactivity from ^{3}H-20:5n-3 into total pentaenoic very long chain fatty acids in both DHA and LNA fed animals and hexaenoic very long chain fatty acid in a LNA fed animals was decreased after light exposure. This light induced decreased synthesis of very long chain fatty acid may be associated with the finding of light induced loss of these fatty acids from the photoreceptor (Chapter V). Since rhodopsin bound lipid contains more polyenoic long and very long chain fatty acids (Aveldano, 1988), the decreased labeling in very long chain fatty acid may be related with rhodopsin function.

One more interesting finding in this experiment was that light stimulated active formation of very long chain fatty acid of n-6 tetraenes and n-3 pentaenes compared to n-6 pentaenes and n-3 hexaenes. This data supports the findings in Chapter V. Together with a higher incorporation of ^{3}H-20:5n-3 into pentaenoic very long chain fatty acid, this data suggests that the metabolic pathway involved in pentaenoic very long chain fatty acid is more active and may play an important role in retinal function.

In summary, this study shows that ^{3}H-22:6n-3 was incorporated directly into photoreceptors without further metabolism whereas, ^{3}H-20:5n-3 and its elongated product C22:5n-3 was a metabolically active precursor for synthesis of very long chain fatty acids. Dietary fat modulates the incorporation of ^{3}H-22:6n-3 and ^{3}H-20:5n-3 into long and very long chain fatty acids in retina. Light interrupts the incorporation of the above substrates into C22:6n-3 and pentaenoic and hexaenoic very long chain fatty acids. Thus, both diet and light are strong determinants of membrane fatty acid distribution to maintain an optimal microenvironment.

On the basis of the findings in this study with previous finding (Voss et al., 1991), a metabolic pathway for very long chain fatty acid biosynthesis in retina is proposed in Figure VIII-11.

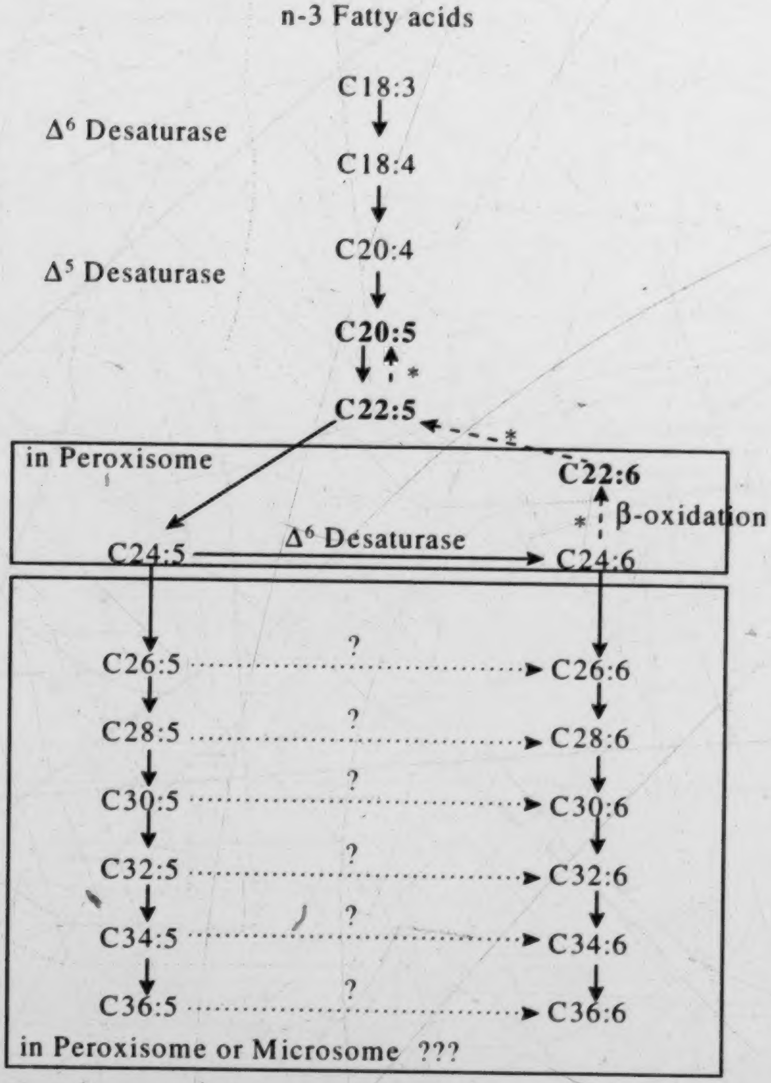
n-3 Fatty acids
C18:3
Δ6 Desaturase
C18:4
C20:4
Δ5 Desaturase
C20:5
*
C22:5
*
in Peroxisome
C22:6
*
β-oxidation
C24:5
Δ6 Desaturase
C24:6
C26:5
C26:6
C28:5
C28:6
C30:5
C30:6
C32:5
C32:6
C34:5
C34:6
C36:5
C36:6
?
in Peroxisome or Microsome ???

Figure VIII-11. Proposed schematic metabolic pathway of n-3 very long chain fatty acids in phosphatidylcholine in retina *in vivo*. C22:6n-3 is not involved in synthesis of very long chain fatty acids. However, C20:5n-3 is elongated to C22:5n-3 and followed by Sprecher's new pathway. The intermediate C24:5n-3 and C24:6n-3 produced maybe give rise to very long chain fatty acid by elongation. Pentaenoic C26 to 36 fatty acid may also give rise to hexaenoic C26 to C36 fatty acid by Δ^6 desaturase enzyme. However, the cellular location of synthesis is not known. * represents retroconversion pathway.

Chapter IX. CONCLUSIONS AND DISCUSSION

The hypotheses listed in Chapter II (p. 51-52) have been addressed as follows:

Hypothesis 1. The content of long and very long chain fatty acids of C24 to C36 n-6 and n-3 fatty acids can be altered by dietary intake of a balanced ratio of n-6/n-3 fatty acids in normal and diabetic animals.

A higher content of n-3 long chain and very long chain fatty acids in phospholipid was found in both normal and diabetic animals fed a high n-3 fatty acid diet (**hypothesis 1-1, Chapter IV & Chapter V**). Specifically, diets high in n-3 fatty acids increased the level of C22:5n-3 and C22:6n-3, while decreasing the C22:5n-6 level in all major phospholipid classes. Feeding a high n-3 fatty acid diet also significantly reduced n-6 tetraenoic very long chain fatty acid in all phospholipids. Unexpectedly, hexaenoic very long chain fatty acids did not respond to dietary high n-3 fatty acid. The effect of diabetic state on changes in membrane fatty acid composition was minor (**hypothesis 1-2, Chapter IV**). These minor changes in fatty acid composition may be due to the short period of diabetes and/or higher amount of essential fatty acid in the diet.

Hypothesis 2. The profile of long and very long chain fatty acids of C24 to C36 n-6 and n-3 fatty acids will be altered by light exposure and can be affected by dietary intake of a balanced ratio of n-6/n-3 fatty acids.

Light exposure of the rod outer segment induced reductions in the level of C22:6n-3 as well as n-6 and n-3 very long chain fatty acids in phosphatidylcholine and phosphatidylserine in animals fed a high n-3 fatty acid diet (**Chapter V**). Within the same n-series of fatty acids, light exposure produced more n-6 tetraenes and n-3 pentaenes than n-6 pentaenes and n-3 hexaenes. Feeding a diet high in n-3 fatty acid exhibited the highest ratio of n-3 pentaenoic to hexaenoic very long chain fatty acids. This result suggests that n-3 pentaenoic very long chain fatty acids are more responsive to diet and light treatment. The free fatty acid and triglyceride fractions contain increased levels of both C20:4n-6 and C22:6n-3 after light exposure. This indicates that the free fatty acid and triglyceride fractions are involved in the metabolism of the C20:4n-6 and C22:6n-3 after light exposure.

Hypothesis 3. The function of rhodopsin can be altered by dietary intake of a balanced ratio of n-6/n-3 fatty acids.

Dietary fatty acid altered rhodopsin content and its functions following light exposure of the rod outer segment (**Chapter V**) or entire retina (**Chapter VI**). Dark adapted rhodopsin content in both rod outer segment and whole retina in animals fed a low n-3 fatty acid diet was higher than that of animals fed a high n-3 diet. On exposure of the visual cells to light, rhodopsin in animals fed a high n-3 fatty acid diet disappear in a relatively short time (**hypothesis 3-1**). After light exposure, the group fed the low n-3 fatty acid diet lost more rhodopsin content compared to animals fed a high n-3 fatty acid diet. Rhodopsin phosphorylation level *in vitro* was proportional to rhodopsin level in rod outer segment (**Chapter V**). Rhodopsin regeneration measured *in vitro* was increased by feeding a high n-3 fatty acid diet (**hypothesis 3-2, Chapter VI**).

Hypothesis 4. Developmental profile of long and very long chain fatty acids of C24 to C36 n-6 and n-3 fatty acids will increase with age in growing animals and will be affected by dietary fat intake.

During rod outer segment development, C22:6n-3 increased and C20:4n-6 decreased in major phospholipids. N-6 tetraenoic acid and n-3 pentaenoic and hexaenoic very long chain fatty acid present in phosphatidylcholine also increased with increasing age (**Chapter VII**). Rats fed both C20:4n-6 and C22:6n-3 exhibited higher levels of C20:4n-6 and C22:6n-3 in phosphatidylcholine in rats at all ages examined (**hypothesis 4.3**). Diets containing C22:6n-3 influenced the level of C22:6n-3 while reducing C20:4n-6. Total n-3 very long chain fatty acid level was higher after feeding DHA (**hypothesis 4.2**). Feeding C20:4n-6 or C22:6n-3 increased the level of n-6 or n-3 very long chain fatty acids, respectively (**hypothesis 4.1 & 4.2**). After weanling. increased C18:3n-3 in diet does not increase the level of C22:6n-3 and very long chain fatty acids present in growing visual cells (**hypothesis 4.4**). The rhodopsin content in retina increased with age. The highest rhodopsin level occurred in the retina of animals fed diets containing C20:4n-6 and /or C22:6n-2. The kinetics of rhodopsin disappearance after light exposure is greatest in animals fed C22:6n-3 (**hypothesis 4.5**).

Hypothesis 5. The synthesis of long and very long chain fatty acids of C24 to C36 n-6 and n-3 fatty will be altered by dietary intake of a balanced ratio of n-6/n-3 fatty acids.

^{3}H-22:6n-3 remained in C22:6n-3 (90% of total), whereas ^{3}H-20:5n-3 was actively incorporated into pentaenoic and hexaenoic very long chain fatty acids (**hypothesis 5-1**). C22:5n-3 derived from ^{3}H-20:5n-3 is among the most highly labeled fatty acid. Light treatment reduced ^{3}H-20:5n-3 incorporation into C22:6n-3 in rats fed a diet containing C22:6n-3. Animals fed the LNA diet exhibited a higher rate of incorporation of ^{3}H-20:5n-3 into n-3 pentaenoic very long chain fatty acid compared to animals fed the DHA diet.

In the photoreceptor cell, the integrity of membrane architecture is fundamental for conversion of light into nervous excitation. The retina contains a high level of docosahexaenoic acid and very long chain fatty acids of carbon chain length longer than C24 with 4, 5, and 6 double bonds (**Chapter IV, V & VII**). On average, over half of the fatty acid in the rod outer segment is docosahexaenoic acid in phosphatidylethanolamine and phosphatidylserine. Approximately 7 % (w/w) is very long chain fatty acids found exclusively in phosphatidylcholine. Changing the exogenous source of essential fatty acids alters the levels of polyunsaturated fatty acids during retinal development and alters visual pigment function.

The data presented in this thesis extends current understanding of the metabolism of fatty acids in rat photoreceptors by feeding diets that reflect a range of dietary fat intake typical of human fat intake. The important conceptual contributions to current understanding are as follows:

1. Dietary fat alters long and very long chain fatty acid constituents of photoreceptor membrane phospholipids.
2. Reciprocal replacement occurs when high n-3 fatty acid diet is fed causing C20:4n-6 and C22:5n-6 to be replaced by C22:6n-3 and n-6 tetra and pentaenoic very long chain fatty acids to be replaced by n-3 pentaenoic acids.
3. Hexaenoic n-3 very long chain fatty acids are not influenced by dietary n-3 fatty acids.
4. Light induces the loss n-6 and n-3 very long chain fatty acids.
5. Within the same n-series, different degrees of unsaturated very long chain fatty acids respond differently to light treatment.
6. A high n-3 fatty acid diet protects against light induced rhodopsin loss and rhodopsin phosphorylation.
7. A high n-3 fatty acid diet significantly affects rhodopsin photolyzing kinetics and rhodopsin regeneration during light exposure.
8. The transition from n-6 towards n-3 long and very long chain fatty acids is characteristic in developing photoreceptors.
9. C20:5n-3 and C22:5n-3 but not C22:6n-3 are the precursors for the synthesis of n-3 very long chain fatty acids

Considerable attention has focused on dietary polyunsaturated fatty acid composition because of the dynamic capability to alter membrane fatty acid composition. However, the effect of dietary intervention on membrane content of these fatty acids has not been studied nor is the effect of diet fat on membrane composition in the retina known. The data in this thesis provide the first experimental evidence for diet induced alteration of long and very long chain fatty acid constituents of photoreceptor membrane phospholipids. This data demonstrates that ROS is in a dynamic state and undergoes significant membrane fatty acid turnover. This turn over could result from fatty acid incorporation during the phospholipid deacylation-reacylation cycle. Both of these metabolic pathways may be important for maintaining appropriate membrane components in specialized cells and may be responsible for supplying components for renewal of the ROS membrane.

The reciprocal replacement of C20:4n-6 and C22:5n-3 by C22:6n-3 and n-6 tetra and pentaenoic very long chain fatty acids by n-3 pentaenoic acids may be important to the study of n-3 fatty acid deficiency. Several studies report functional deficits in vision such as vision loss, abnormal electroretinogram in rats, monkeys and human infants fed n-3 deficient diets (Neuringer et al., 1986; Uauy et al., 1990 & 1992). The diet induced increase in C22:6n-3 may provide insight into beneficial effects of high n-3 diets in retinal disease such as retinitis pigmentosa where plasma and retina level of C22:6n-3 are low. The type of change observed in the fatty acid composition of phospholipids presented in this thesis could affect function of the retina and have potential relationships to clinical disorders.

Docosapentaenoic and docosahexaenoic acid are strong candidates for the synthesis of very long chain fatty acid, since these two fatty acids are generally considered to be the major end products of n-6 and n-3 fatty acid metabolism. However, the novel finding of this thesis indicates that docosahexaenoic acid is not a precursor for the synthesis of n-3 very long chain fatty acids. Our results suggest that retina has a mechanism for selective uptake of C22:6n-3 through direct acylation. C20:5n-3 and C22:5n-3 were the preferential substrates for desaturase and elongase enzymes during very long chain fatty acid synthesis. As the cellular location for synthesis of very long chain fatty acid is not known, the answer of whether enzyme systems for these fatty acids are located in the peroxisome or the microsome remains to be an issue concerning synthesis of very long chain fatty acids.

If very long chain fatty acids have an essential function, the precursor molecule of these fatty acid should be identified. C20:5n-3 but not C22:6n-3 appear to be central to the synthesis of very long chain fatty acids. Since C20:5n-3 has been ignored in current infant formula, our finding suggests that supplementation of this fatty acid should be considered.

Dietary fat clearly influenced rhodopsin function (Chapter V & VI). Feeding a low n-3 fatty acid increased the amount of rhodopsin resulting in higher rhodopsin phosphorylation. However, the loss after light exposure was greater when a low n-3 fatty acid diet was fed. This suggests that the higher content of C22:6n-3 and increased pentaenoic very long chain fatty acids after feeding a high n-3 fatty acid diet (Chapter V) are protective against the loss rhodopsin induced by light.

Feeding a high n-3 fatty acid diet to animals has significant effects on rhodopsin photolyzing kinetics following light exposure. The disappearance of rhodopsin in a relatively short time during light exposure occurs after feeding a high n-3 fatty acid diet. This may be involved in more regeneration/recycling of rhodopsin. This thesis presents experimental evidence implying that fatty acid is an important determinant for rhodopsin regeneration in the retina. It would, therefore, be logical to follow up these observations by measuring the effect of diet fat on some of the rhodopsin mediated parameters involved in membrane signal transduction such as cGMP, phosphatidyldiesterase and rhodpsin kinase.

The structural and functio[illegible]haracteristics of retinas are sensitive to light exposure. The data presented in this [illegible]esis demonstrate for the first time light induced changes in very long chain fatty acids. Light exposure caused reduction in C22:6n-3, n-6 and n-3 very long chain fatty acids. Light induced reduction in C20:4n-6 and C22:6n-3 is mediated by phospholipase A_2 (Birkle & Bazan, 1989; Reinboth et al., 1996). Thus, the reduction of very long chain fatty acids following light exposure suggests that light also stimulates phospholipase A_1 in photoreceptor cells.

One of the interesting findings of this experiment is that within the same n-series, different degrees of unsaturation respond differently to light treatment. For example, light exposure activates more n-6 tetraenes and n-3 pentaenes than n-6 pentaenes or n-3 hexaenes. N-3 pentaenoic very long chain fatty acids respond more to diet and light treatment. Although the implication of this finding is not known, further research is required to identify the functional role of each n-6 and n-3 very long chain homologue. The effect of tetraenoic very long chain fatty acid has been explored in membrane signal transduction in human neutrophills and rat brain (Hardy et al., 1994 & 1995). It would be of interest to measure signal transduction in photoreceptor cells by using individual n-6 and n-3 very long chain fatty acid homologues which can be easily modulated by the dietary fat source.

Development of the visual system follows a sequence of events, many of which involve synthesis of complex lipids to provide basic membrane structural components. Inclusion of a small amount dietary fat is powerful enough to change membrane long and very long chain fatty acids in the developing photoreceptor of retina. This suggests that the developing retina is vulnerable to exogenous nutrient supply. Altered membrane fatty acid composition by diet may have significant implications for early retinal development related to rhodopsin function and visual cell renewal. In this regard, the choice of infant formula for infants may be important for visual cell maturation and visual function. Although it is challenging to carry out a follow up study, it would be interesting to design experiments to determine whether early exposure to diet is related with later function of visual cells.

The transition from n-6 toward n-3 long and very long chain fatty is characteristic in the developing photoreceptor cells. N-6 tetraenoic and n-3 pentaenoic and hexaenoic acid increased with age. Since rhodopsin content also increased during this period, these transitions may be strongly related to rhodopsin function. These findings imply that a critical period exists during which fatty acid is important. It would be interesting to measure retinal parameters such as rod outer segment length, photoreceptor cell numbers and outer nuclear layer thickness in growing animals following the same feeding protocol as used in the thesis.

It is generally known that infants born prematurely or with a very low birth weight are at risk for visual impairment. It is not known whether this visual impairment is due to delayed development that is reversible. The findings in this thesis suggest that the light environment in which the infants are raised is another important risk factors in infant visual development. Since photoreceptor membranes are damaged easily even in the low levels of light *in vivo* and the major steps of visual development occur during the first 18 months of life in the human, it is apparent that providing optimum light intensity in the clinical setting is important.

From all of findings made in this thesis, it is evident that n-3 fatty acids are important in visual cells as a structural component for providing the optimal function of rhodopsin. However, these studies were carried out on animals in disease free states. It will be fascinating to extend the experimental design to retinal disease such as retinitis pigmentosa which has several different types of animals models. Of interest is whether the characteristic low level of C22:6n-3 in plasma and retina of retinitis pigmentosa can be increased by dietary high n-3 fat. It is also interesting to study this disease model to determine if the finding that n-3 hexaenoic very long chain fatty acids are not influenced by dietary n-3 fat (Chapter IV & V). This thesis generates clear evidence that dietary fat is a strong determinant of the retinal fatty acid composition and rhodopsin function. Dietary fat could be an important tool for therapy.

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Table A-1. Composition of semipurified diet

Component	Composition (g per kg)
High-protein casein	270.5
Corn starch	378.0
Non-nutritive cellulose	80.0
Vitamin mix [1]	10.0
Mineral mix [2]	50.0
Choline	2.75
Inositol	6.25
L-methionine	2.5
Fat mix	200.0

[1] Vitamin mix (A.O.A.C.) [2] Mineral mix (Bernhart-Tomarelli) The composition of basal diet, 1 and 2 was reported in detail in Clandinin (1978). Vitamine A mixture (9,900 IU) composed of 50% retinal acetate and 50% retinyl palmitate. Vitamine E (60 IU) in the form of D,L- α- tocopherol acetate.

Table A-2. Composition of basal diet

Component	Composition (g per kg)
Casein	270.0
Starch	200.0
Glucose	207.65
Non-nutritive cellulose	50.0
Vitamin mix [1]	10.0
Mineral mix [2]	50.85
Choline	2.75
Inositol	6.25
L-methionine	2.5
Fat mix	200.0

[1] Vitamin mix (A.O.A.C.) [2] Mineral mix (Bernhart-Tomarelli) The composition of basal diet, 1 and 2 was reported in detail in Clandinin (1978). Vitamine A mixture (9,900 IU) composed of 50% retinal acetate and 50% retinyl palmitate. Vitamine E (60 IU) in the form of D,L- α- tocopherol acetate.

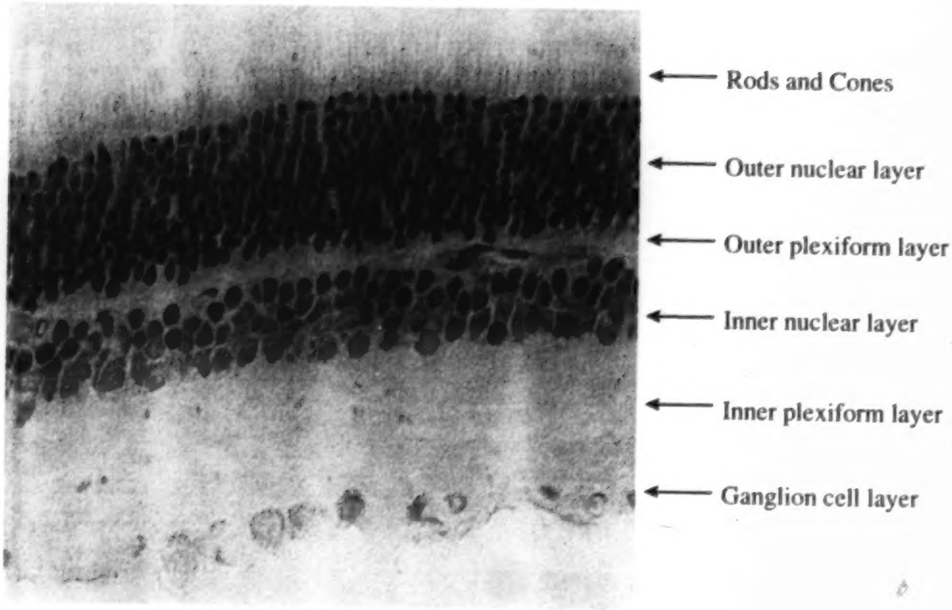
Rods and Cones
Outer nuclear layer
Outer plexiform layer
Inner nuclear layer
Inner plexiform layer
Ganglion cell layer

Figure A-1. Cross section of rat retina. (Magnification X625)

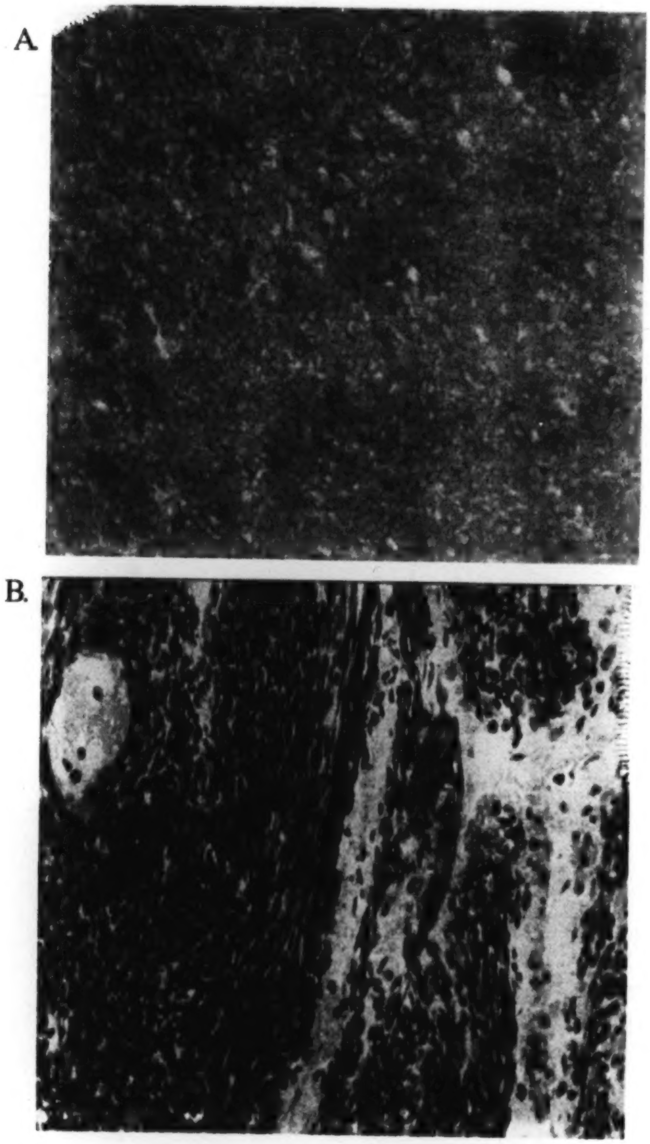

Figure A-2. Cross section of rat rod outer segment and retinal cell body. A. Rod outer segment (homogenized). B. Retinal cell body after rod outer segment is separated from retina. (Magnification X625)

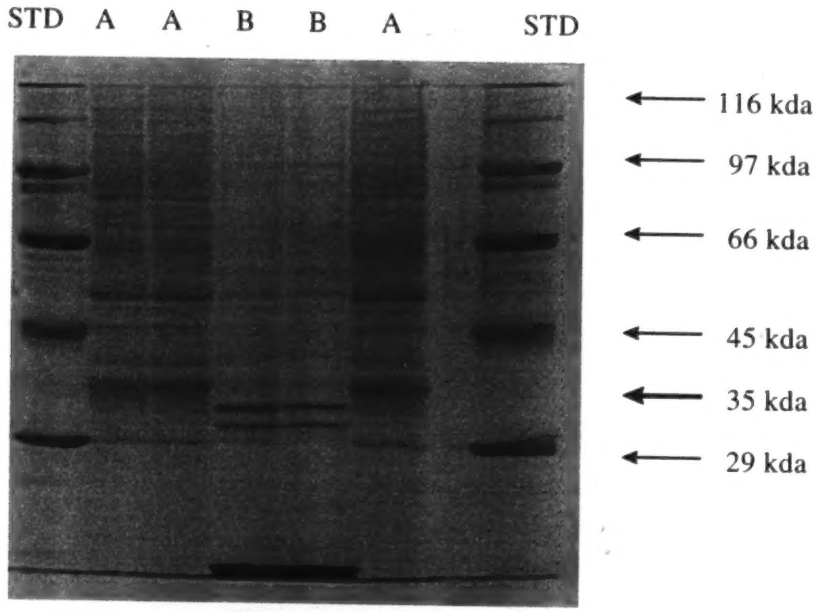
STD
A
A
B
B
A
STD
116 kda
97 kda
66 kda
45 kda
35 kda
29 kda

Figure A-3. Polyacrylamide gel electrophoresis of rod outer segment (A) and retinal cell body (B). Molecular weight of opsin is 35 kda. Molecular weight of protein standards (STD): 29 kda, carbonic anhydrase; 45 kda, ovalbumin; 66 kda, bovine albumin; 97 kda, phosphorylase b; 116 kda, β-galactosidase. Proteins are visualized with Commassie Blue stain.

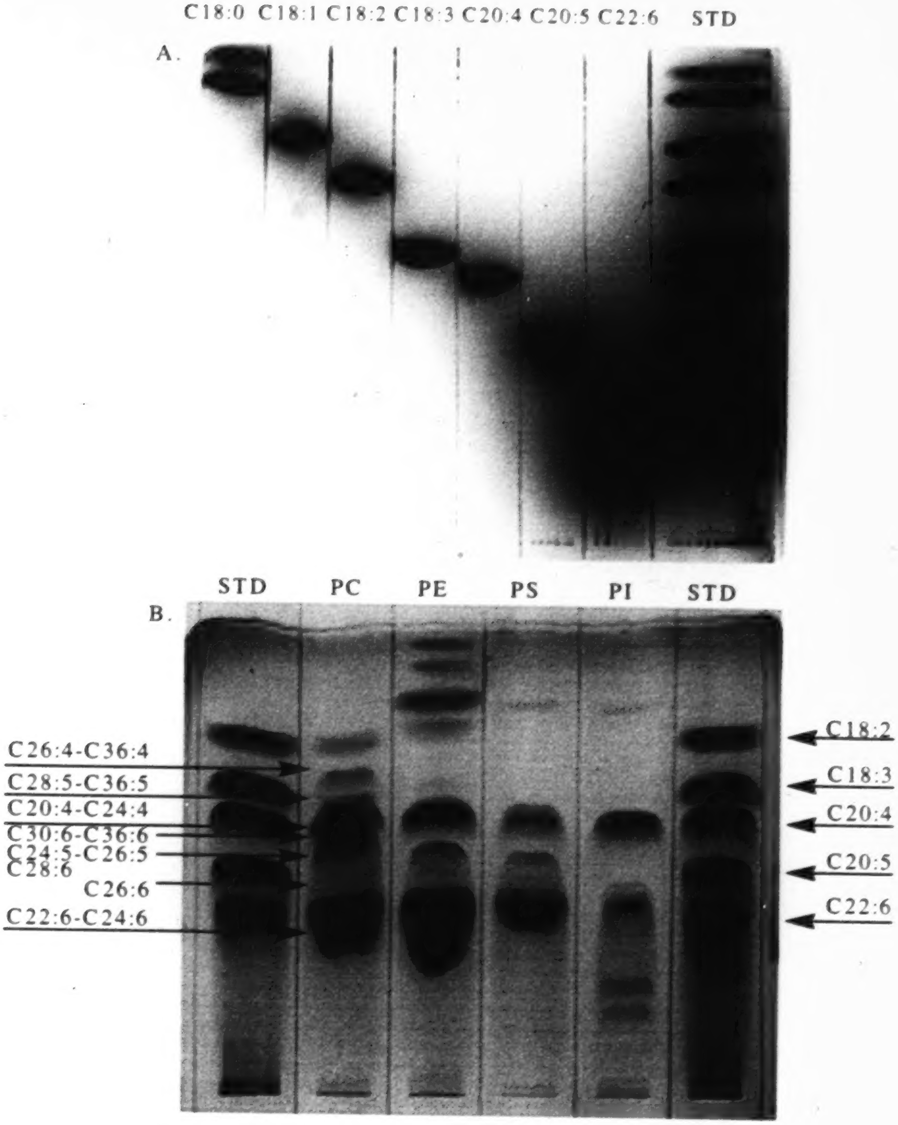
C18:0 C18:1 C18:2 C18:3 C20:4 C20:5 C22:6 STD
A.
STD PC PE PS PI STD
B.
C26:4-C36:4
C28:5-C36:5
C20:4-C24:4
C30:6-C36:6
C24:5-C26:5
C28:6
C26:6
C22:6-C24:6
C18:2
C18:3
C20:4
C20:5
C22:6

Figure A-4. Typical chromatogram of fatty acid methyl esters by argentation thin layer chromatography of standard (A) and retinal phospholipids (B). Each sample in A represents 40 μg of standard methyl esters (Sigma Chemical Co., Canada). Each phospholipid in B is prepared from 20 rat retinas. Silica gel H plate was impregnated with 10% of silver nitrate ($AgNO_3$) solution, activated for 1 hour at 110°C and developed in a solvent system of hexane:diethyl ether:acetic acid:toluene:acetone (50:4:2:40:4, by vol) for 1 hour and then 40 min for a second development. The plate was charred to visualize the fatty acids separated.

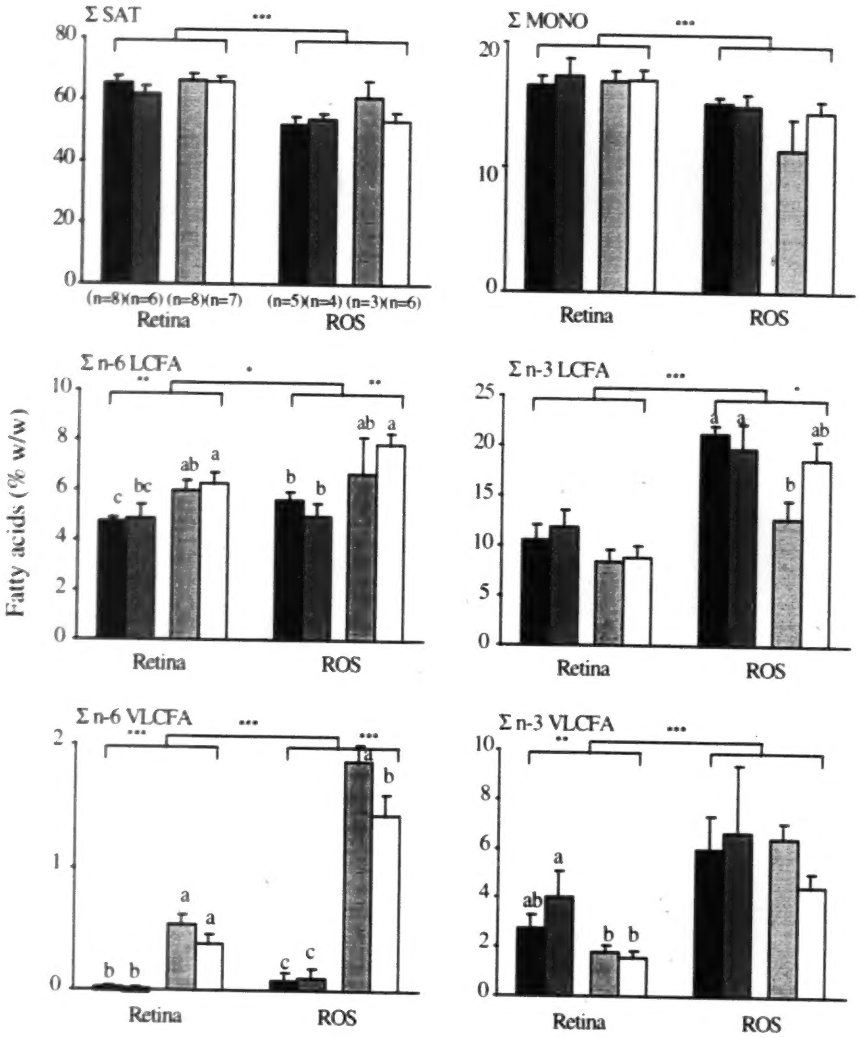

Figure A-5. Comparison of fatty acid concentration in phosphatidylcholine of retina and rod outer segment. Values given are means (listed in ΣSAT) ± S.E.M. Significant difference between retina and rod outer segment was analyzed by three way analysis of variance procedure: *, $p<0.05$, ***, $p<0.001$. Values without a common superscript in each retinal sample indicate a significant effect of diet: *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$. Abbreviations used: ROS, rod outer segment; ΣSAT, sum of saturated fatty acids; ΣMONO, sum of monounsaturated fatty acids; Σn-6 LCFA, sum of n-6 long chain fatty acids; Σn-3 LCFA, sum of n-3 long chain fatty acids; Σn-6 VLCFA, sum of n-6 very long chain fatty acids; Σn-3, sum of n-3 very long chain fatty acids. ■, control animals fed a high n-3 fatty acid diet; ■, diabetic animals fed a high n-3 fatty acid diet; ▨, control animals fed a low n-3 fatty acid diet; □, diabetic animals fed a low n-3 fatty acid diet.

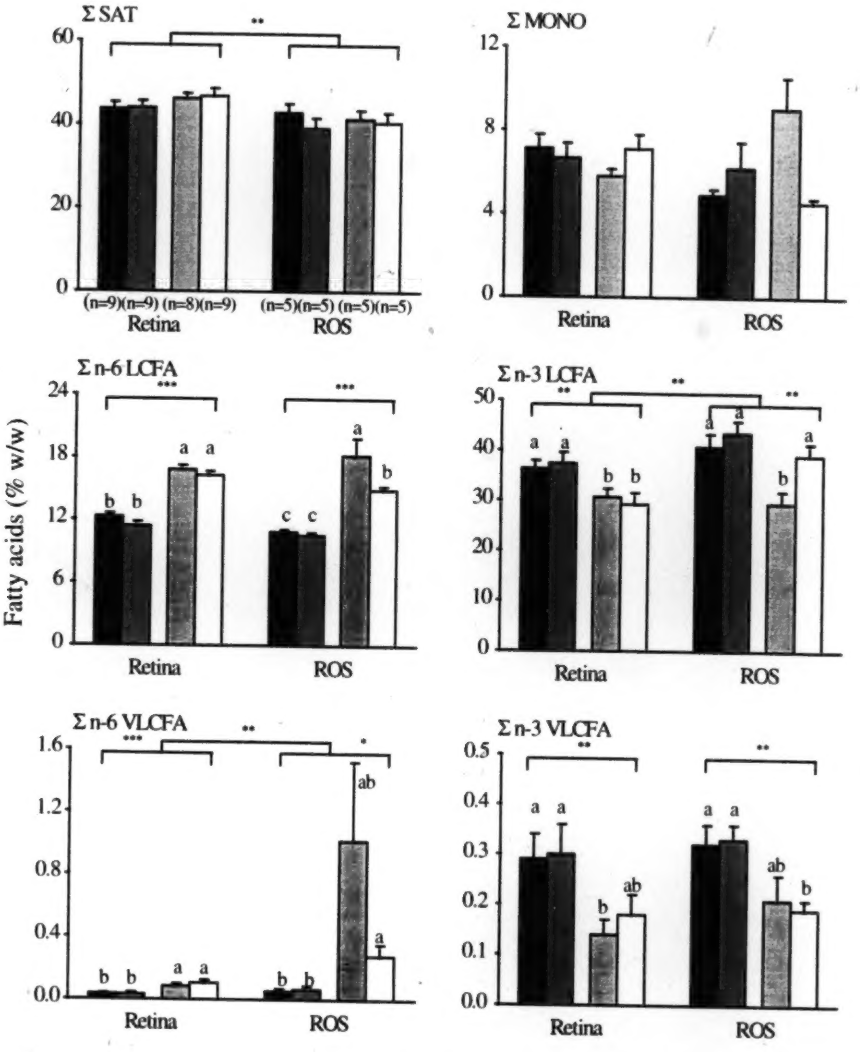

Figure A-6. Comparison of fatty acid concentration in phosphatidylethanolamine of retina and rod outer segment. Values given are means (listed in ΣSAT) ± S.E.M. Significant difference between retina and rod outer segment was analyzed by three way analysis of variance procedure: **, p<0.01. Values without a common superscript in each retinal sample indicate a significant effect of diet: *, p<0.05, **, p<0.01, ***, p<0.001. Abbreviations used: ROS, rod outer segment; ΣSAT, sum of saturated fatty acids; ΣMONO, sum of monounsaturated fatty acids; Σn-6 LCFA, sum of n-6 long chain fatty acids; Σn-3 LCFA, sum of n-3 long chain fatty acids; Σn-6 VLCFA, sum of n-6 very long chain fatty acids; Σn-3, sum of n-3 very long chain fatty acids. ■, control animals fed a high n-3 fatty acid diet; ■, diabetic animals fed a high n-3 fatty acid diet; ▨, control animals fed a low n-3 fatty acid diet; ☐, diabetic animals fed a low n-3 fatty acid diet.

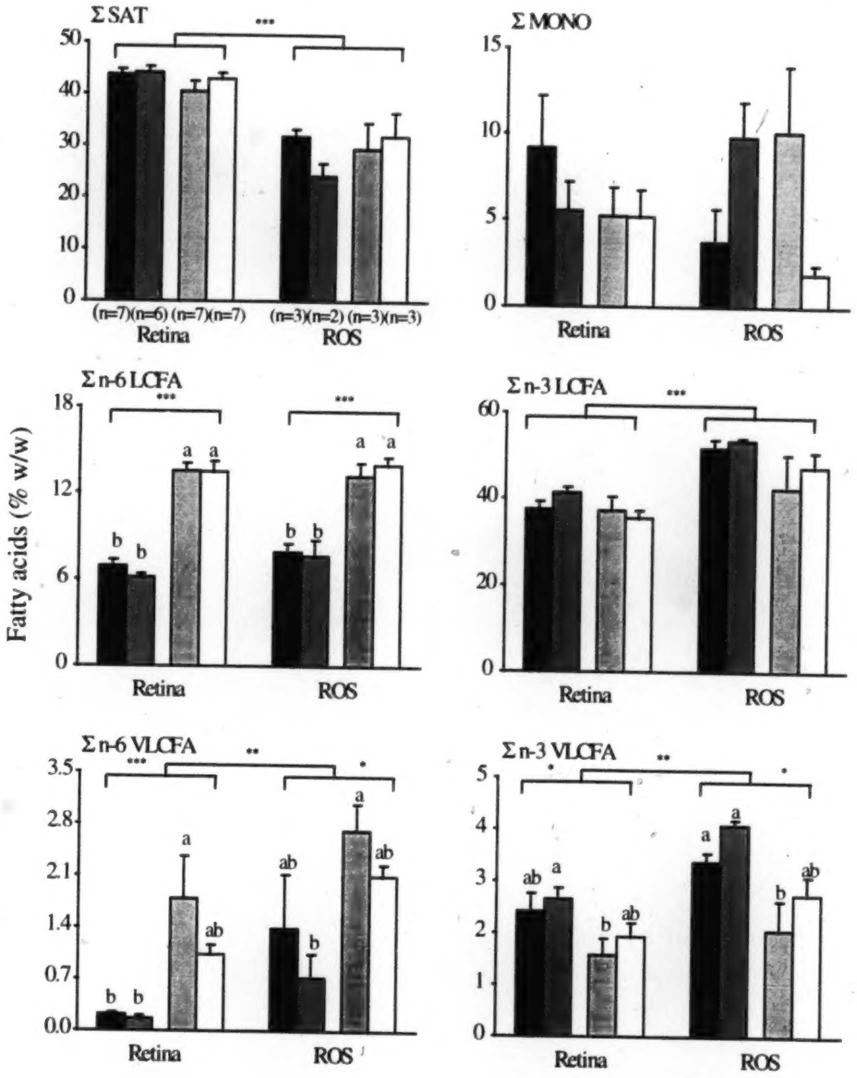

Figure A-7. Comparison of fatty acid concentration in phosphatidylserine of retina and rod outer segment. Values given are means (listed in ΣSAT) ± S.E.M. Significant difference between retina and rod outer segment was analyzed by three way analysis of variance procedure: **, p<0.01, ***, p<0.001. Values without a common superscript in each retinal sample indicate a significant effect of diet: *, p<0.05, ***, p<0.001. Abbreviations used: ROS, rod outer segment; ΣSAT, sum of saturated fatty acids; ΣMONO, sum of monounsaturated fatty acids; Σn-6 LCFA, sum of n-6 long chain fatty acids; Σn-3 LCFA, sum of n-3 long chain fatty acids; Σn-6 VLCFA, sum of n-6 very long chain fatty acids; Σn-3, sum of n-3 very long chain fatty acids. ■, control animals fed a high n-3 fatty acid diet; ▩, diabetic animals fed a high n-3 fatty acid diet; ▨, control animals fed a low n-3 fatty acid diet; □, diabetic animals fed a low n-3 fatty acid diet.

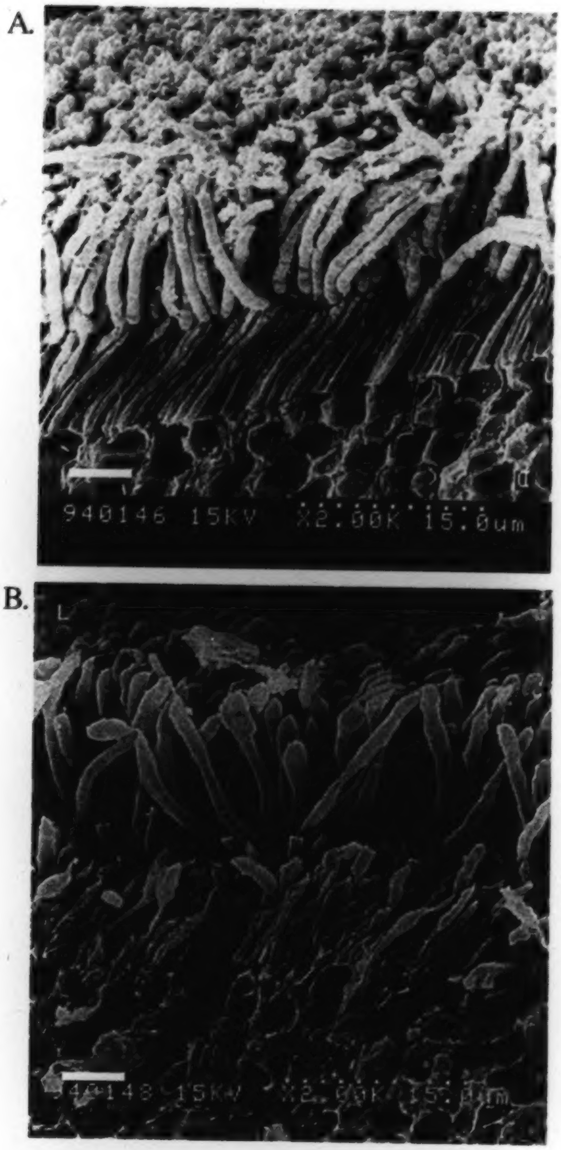
A.
940146 15KV X2.00K 15.0um
B.
L
940148 15KV X2.00K 15.0um

Figure A-8. Scanning electron micrograph (SEM) of rat retina of either dark adapted (A) or light exposed (B) for 48 hours after feeding a low n-3 fatty acid diet. Bar represents 5.5 µm. Both rod outer segment and inner segments are disorganized and swollen in animals exposed to light compared to animals kept in the dark.